Evaluation of gamete dysfunction as a cause of failed human *in vitro* fertilization.

by

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Declaration

I the undersigned declare that the work contained in this dissertation is my own original work and has not previously in its entirety or in part been submitted at any university for a degree

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ABSTRACT

Chapter 1 provides literature based background information on the clinical importance of sperm morphology as recorded by strict criteria during the diagnostic approach of the infertile couple. Furthermore, the use of a sequential diagnostic schedule for couples in an assisted reproductive programme is emphasized. The author revisited the literature on chromatin packaging of spermatozoa and addresses this issue as an additional semen parameter providing information relating to DNA damaged spermatozoa. The chapter also includes evidence underlining the growing need for the implementation of the acrosome reaction as an important contribution to the assisted reproductive programme. Chapter 2 provides detailed descriptions of the material and methods used during the study. Chapter 3 is sub-divided into 5 sections, each of which represents a separate study that was prepared as a scientific paper. The study included 338 couples consulting for infertility treatment at various gynaecologists in Pretoria and Johannesburg. The diagnostic assisted reproductive laboratory support was provided by the Andrology laboratory of Drs du Buisson and partners from Pretoria. In the first study the role of chromatin packaging as an indicator of in vitro fertilization rates, the semen samples from 72 men were used to record their chromatin packaging quality as well as their sperm morphology classification. Significant different percentages CMA_3 staining (mean±SE) were recorded among the 2 morphology groups, namely 65.9%±3.5 and 44.5%±1.7 ($p=0.001$). Using cut off values for chromatin packaging established during the first study, the second study utilized semen from 140 men

in the *in vitro* fertilization (IVF) and intracytoplasmic sperm injection programme (ICSI) to analyze for sperm concentration, motility, morphology and chromatin packaging (CMA_3). IVF and ICSI data were stratified using 3 basic cut off values for CMA_3 staining, namely <44%, >44-60% and >60%. The study concluded that results on the chromatin packaging quality of spermatozoa could be used as an additional parameter of sperm quality since it could provide valuable information on decondensation status of a given sperm population. The third study aimed to establish zona pellucida induced acrosome reaction response (ZIAR) among 35 couples with normal and G-pattern sperm morphology and repeated poor fertilization results during assisted reproduction treatment. Interactive dot diagrams, divided patients into 2 groups i.e. ZIAR<15% and ZIAR>15% with mean fertilization rates of 49% and 79%, respectively. The study concluded that the ZIAR test has diagnostic potential, since it can assist the clinician to identify couples that will benefit from ICSI therapy. The forth study revisited the importance of micro-assay for acrosome reaction determinations in a diagnostic andrology laboratory. The micro-assay not only allows the use of a single zona pellucida, but also facilitates the future possibility of using recombinant zona pellucida proteins in a diagnostic test system. The final study in Chapter 3 includes results obtained from 49 couples (172 oocytes) and aimed to evaluate the role of chromatin packaging and sperm morphology during sperm-zona binding, sperm decondensation and the presence of polar bodies among 170 oocytes that failed in vitro fertilization (IVF). Odds ratio analyses indicated that being in the a group with elevated CMA_3 staining i.e. >60%, the risk of

decondensation failure increases 15.6 fold relative to normal CMA_3 staining <44%. Chapter 4 underlines the validity of the sequential diagnostic approach and summarizes the results and value of a multistep diagnostic scheme. The chapter concludes with the recommendation that both chromatin packaging quality and zona pellucida mediation of the acrosome reaction should be part of the diagnostic tools in the assisted reproductive programme.

OPSOMMING

Die literatuuroorsig in Hoofstuk 1 konsentreer in hoofsaak op die kliniese belang van sperm morfologie en die uitbreiding van die diagnostiese toetse en hantering van die egpaar in die reproduktiewe ondersteuningsprogram. Die kromatien pakkingskwaliteit van die spermsel word onderskryf as 'n belangrike toevoeging tot die diganostiese program, aangesien DNS skade dikwels saam met kromatiendefekte aangetref word. Die rol van die akrosoomreaksie word ook in detail literatuuroorsigtelik beklemtoon. Hoofstuk 2 bevat volledige inligting omtrent materiaal en metodes wat in die studie gebruik is. Hoofstuk 3 bevat die eksperimentele gegewens wat in 5 afsonderlike sub-afdelings as wetenskaplike publikasies aangebied word. Die studies bestaan uit data van 338 pasiënte, wat deur verskillende ginekoloë van Pretoria en Johannesburg gekonsulteer is waartydens drs. du Boisson en vennote van Pretoria die diagnostiese reproduktiewe laboratoriumdienste verskaf het. Die eerste studie stel dit ten doel om die belang en korrelasie van die spermsel kromatienpakkingskwaliteit van 72 mans te vergelyk met die morfologiese bou van sie sel. Aangesien morfologie reeds gevertig is as 'n kliniese voorspeller van bevrugting was dit nodig om hierdie parameter te vergelyk met die kromatienpakking van die sel. Twee afsnypunte word vir die normo-en teratozoospermiese mans identifiseer naamlik, 44.5%±1.7 en 65.9%±3.5 (p=0.001), respektiewelik. Die tweede studie gebruik die afsnypunte 44% en 66% om die *in vitro* bevrugting en intrasellulêre sperm inspuiting (ICSI) data te ontleed. Die resultate dui aan dat kromatienpakking 'n waardevolle bydrae tot die diagnostiek van die pasiënte lewer. Die derde studie

stel dit ten doel om die waarde van die zona pellucida geinduseerde akrosoomreaksie (ZIAR) te bepaal. Die studie sluit die data van 35 egpare in wat almal normale of G-patroon morfologie het en verder onverklaarde swak bevrugtings resultate tydens in vitro bevrugtingsterapie. Interaktiewe punt diagram (interactive dot diagrams) verdeel die data in twee groepe naamlik, ZIAR<15% en ZIAR>15% met gemiddelde bevrugtingssyfers van 49% en 79%, respektiewelik. Die studie sluit af met die gedagte dat die ZIAR toets 'n groep pasiënte identifiseer met 'n besondere fisiologiese afwyking d.i. subnormale akrosoom respons op zona pellucida blootstelling. Die vierde afdeling van die hoofstuk onderstreep die belang van die mikro-tegniek vir die bepaling van die akrosoom reaksie, wat tydens die projek gebruik is Die vyfde afdeling van Hoofstuk 3 stel dit ten doel om 170 onbevrugde eierselle van 49 pasiënte te ontleed vir moontlike oorsake vir die mislukte bevrugting. Ondersoeke sluit in die kromatienpakking, sperm-zona binding, sperm dekondensasie en die teenwoordigheid van polêre liggaampies. Statisties blyk dit dat indien 'n kromtienpakking nie normaal is nie (>66%) het die spermsel 'n 15 keer groter kans om nie te dekondenseer nie. Hoofstuk 4 bespreek die noodsaaklikheid van die diagnostiese skedule by die hantering van die onvrugbare egpaar in.

CONTENTS

This thesis is dedicated to

Christo, Caro and Junro

"Die weelde van ware liefde
is onskatbaar"
("true love is an infinite luxury")

Without their motivation, support and love
I would not have been able to
successfully complete
this study.

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Preface

The development of diagnostic techniques in andrology, i.e. the sperm-zona binding assays, identification and role of normal spermatozoa, the role of chromatin packaging and DNA breakage on fertilization, has stimulated scientists to focus their research on development a sequential diagnostic schedule to identify fertilization failure during assisted reproduction. It is well recognized that fertilization success or failure cannot be contributed to a single sperm or oocyte defect and that one should rather use a multiple diagnostic approach to identify the cause of the gamete dysfunction.

The overall objectives of the present study are to present new data that will assist the clinician or scientist in the general day-to-day management of male infertility. The specific aims are to validate existing technologies and to develop new diagnostic techniques, such as the micro-volume acrosome reaction assay and determine the predictive value for in vitro fertilization and intracytoplasmic sperm injection outcome. Furthermore, the work aimed to define laboratory conditions to use of a single human zona pellucida to determine the acrosome mediating qualities.

This could be an important contribution to the existing technology regarding acrosome determinations, since most of the published work, describe acrosome reaction techniques that uses large amounts of human zona pellucida. The

scarcity of human zonae therefor has placed the use of a physiological acrosome reaction technique out of reach of most clinical diagnostic laboratories.

Notwithstanding the fact that we are aware of the fact that fertilization can be due to oocyte problems, the present work is based on the hypothesis that human fertilization failure can be due to the presence of one or more sperm defects. The parameters that have previously validated in this regard include sperm morphology as described by Tygerberg strict criteria as well as sperm zona binding evaluations as recorded by the hemizona assay. These determinations have been established for more than a decade as important measurements in male fertility diagnosis.

The present results provide more information regarding DNA chromatin packaging, decondensation and acrosome reaction using a novel assay in a patient population where sperm morphology and sperm-zona binding was used as discriminators fertilization failure and/or success.

CHAPTER 1

INTRODUCTION

Semen quality is conventionally determined according to the concentration, motility and morphology of spermatozoa in the ejaculate. A comprehensive high quality semen analyses is an essential first line investigation for infertile couples. Furthermore, during standard *in vitro* fertilization (IVF), complete failure of fertilization occurs in 10-15% of treatment and although causes may be unclear, many studies indicate that sperm defects appear to be major contributors (Kruger et al., 1986, Liu et al., 1989 a, b, Franken et al., 1993, Oehninger et al., 1997). It is recognized that oocyte immaturity or abnormalities can also contribute to failure of fertilization. Where the majority of oocytes fertilize, the few that do not fertilize often have defects (Bedford and Kin, 1993, Van Blerkom et al., 1994). However, oocyte factors appear to be uncommon causes for complete failure of fertilization. Standard follicle stimulation treatments rarely produce uniformly abnormal or immature oocytes.

Although most of the patients with fertilization failure in IVF can be treated with intracytoplasmic sperm injection (ICSI, Van Steirteghem et al., 1993), diagnosing the causes of fertilization failure is important. Therefore despite the great therapeutic advantages of the technique ICSI, it often provides solutions to clinicians in the absence of an etiological or patho-physiological diagnosis

(Oehninger et al., 1997). Ideally, fertilization failure or success should be detected prior to IVF or ICSI. The evaluation of a single sperm parameter or function may not provide enough power for prediction of fertilization or implantation outcome. This reflects the complexity of events leading to sperm-oocyte interaction and conception. Oehninger et al., (1991) have suggested the development of a sequential, multi-step diagnostic approach for the evaluation of the various structural, dynamic and functional sperm characteristics. This approach has been the result of combined information derived from the basic and clinical areas of the andrology and reproductive endocrinology disciplines. Such a diagnostic scheme should include (i) assessment of the "basic" semen analysis and (ii) functional testing of spermatozoa.

Four categories of tests have been proposed as components of this second level approach: (i) computer-assisted evaluation of sperm motion characteristics (CASA), (ii) inducibility of the acrosome reaction, and bio-assays that sequentially assess gamete interaction including (iii) sperm-zona pellucida binding tests and (iv) sperm-hamster egg penetration assay (Oehninger 1995, WHO 1992, Consensus Workshop 1996). Different laboratories have highlighted the diagnostic power of these tests and the World Health Organization (WHO) has incorporated them under the category of functional tests (WHO 1992). However, as discussed at a recent Consensus Workshop in Advanced Andrology (Consensus Workshop 1996) it was agreed that better standardization of CASA methods and acrosome reaction techniques should be implemented prior to its

introduction as a routine clinical tool (Consensus Workshop 1996). Importantly, among the bio-assays of sperm-egg interaction, it was concluded that because of the powerful evidence for prediction of both fertilization and its failure in the IVF setting, sperm-zona binding tests should be favored among the functional assays (Consensus Workshop 1996). Notwithstanding some practical limitations of these assays, we have incorporated the hemizona assay (HZA) as part of our routine advanced diagnostic scheme. Multiple regression analyses have demonstrated in prospective studies that the HZA results have the highest predictive power for fertilization success/failure among various sperm parameters (Oehninger et al., 1992, Franken et al., 1993, Oehninger 1995). Receiver operating curve (ROC) analysis has confirmed high specificity and sensitivity for HZA results in the prediction of fertilization outcome (Coddington et al., 1994).

Sperm morphology

A systematic study of human gamete interaction in vitro has become feasible since the introduction of in vitro fertilization techniques. Results obtained by these techniques have questioned the predictive value of conventional semen analyses. Also the history of abuse of sperm functional tests has not helped; far too many have been heralded by their advocates as the best diagnostic approach and clinicians have been too quick to pronounce men fertile or infertile on the basis of a single favorite test. The assessment of sperm morphology, one of the three most important parameters (concentration and motility being the

other two) has been reported as a good predictor of fertilization in vitro (Kruger et al., 1986, Oehninger and Kruger 1995). Even though the basic semen parameters are descriptive in nature, several studies obtained good correlation between in vitro fertilization and motility (Robinson et al., 1994) and normal/abnormal sperm morphology (Kruger et al., 1986, Enginsu et al., 1993).

During an evidence based literature study (Coetzee et al., 1998) on sperm morphology the majority of papers (81%) concluded that normal morphology, including acrosomal morphology, played a role in the diagnosis of male fertility potential. The advancement of infertility treatment with the introduction of ICSI procedure has made the correct classification of male fertility paramount, to ensure the optimum cost-benefit ratio. This is especially true in cases of severe male factor infertility. The ICSI procedure has been shown to consistently produce fertilization rates of between 50% to 70% in severe male factor cases. This underlines the importance of being able to identify these cases so that they can be given the option of ICSI, or at least a diagnostic cycle during which half of the oocytes are fertilized by ICSI and the rest inseminated.

Since sperm morphology, at least in our hands, forms the cornerstone of our IVF diagnostic approach; we designed a prospective analytical study using morphology as basis of classification of different groups of couples attending our IVF/ICSI programme.

Chromatin packaging quality

During the last stages of spermiogenesis, elongation and progressive condensation of the chromatin take place, which, with simultaneous acrosome attachment, results in the typical shape of the sperm head. Chromatin condensation is associated with biochemical changes such as replacement of histones which bind to DNA, first by transition proteins and then by arginine-rich protamines and the formation of chromatin-stabilizing disulphide bonds (Zamboni 1987, Green et al., 1994). The high degree of chromatin aggregation protects the mature sperm against physical and chemical damage. It is only within the ooplasma of an activated oocyte that the sperm chromatin becomes decondensed as a result of the cleavage of disulphide bonds and the substitution of protamines by oocyte-derived histones. Due to the tight packaging afforded by the protamines, any modification or absence of these proteins could lead to an anomaly in the packaging process of the sperm nuclei and influence sperm quality and fertilizing capacity. One of the major factors leading to chromatin packaging problems in ejaculated human spermatozoa could be faulty protamine deposition during spermiogenesis (Nikolettos et al., 1999).

An association between abnormal sperm chromatin packaging and the presence of DNA strand breaks has been shown to exist and it has been postulated that these anomalies may arise due to faults in the mechanisms that package and

protects the sperm chromatin during spermiogenesis (Manicardi et al., 1995). Spermatozoa with incomplete chromatin condensation apparently more often display single-stranded rather than double stranded DNA (Pederson 1987) or possess chromosomal abnormalities (Abramsson et al., 1982). It is also known that spermatozoa with abnormal chromatin nuclear chromatin organization are more frequent in infertile men than in fertile (Evenson et al., 1994).

Alterations in sperm chromatin might result in defective decondensation and DNA activation during fertilization, leading to a delay in the formation of the male pronucleus and/or first division. This will cause early embryonic wastage or poor embryonic development (Hamamah et al., 1997). The ability of chromatin to stain with specific dyes and fluorochrome tp DNA can give indications to the packaging quality of the chromatin, which occurs during spermiogenesis. Several techniques has been described such as the Sperm Chromatin Structure Assay (SCSA) which uses the metachromatic properties of acridine orange, other dyes include the use of aniline blue (Henkel et al., 1994) and fluorochromes, for example, methyl green (Godowicz 1977), giemsa stain (Windt et al., 1994), ethidium bromide (Filatov et al., 1999), acridine orange (Evenson et al., 1999), and chromomycin A_3 (Bianchi et al., 1996) (CMA_3). The lower chromatin packaging quality in morphologically normal sperm may represent a major limiting factor in the fertilizing ability of male factor patients. A high percentage of cases with an increased CMA_3 staining was reported in certain forms of male factor infertility, which underlined the ability of CMA3 staining to distinguish

separate populations among morphologically normal spermatozoa (Bianchi et al., 1996)

Sakkas et al (1996) investigated whether morphology and chromatin anomalies in human spermatozoa can influence fertilization after ICSI. They examined unfertilized oocytes and assessed sperm chromatin packaging. Although they did not postulate that the fertilization failure was entirely due to sperm defects, it seems likely that poor chromatin packaging and/or damaged DNA may contribute to failure of sperm decondensation after ICSI and to a resultant failure in fertilization. The quality of sperm chromatin (DNA) is an important factor in the fertilization process and is especially critical where one spermatozoon is artificially selected.

According to the available data, sperm decondensation defects and DNA anomalies may results in unrecognized de-arrangements of spermatozoa, regardless of sperm morphology which is known to influence the fertilization. Consequently in cases of severe male factor infertility, studies of sperm DNA status are indicated (Nikolettos et al., 1999).

Acrosome reaction

In all mammals, sperm cells are required to fertilize oocytes, thereby providing a haploid set of chromosomes with a paternal pattern of genomic imprinting

needed for normal development and triggering oocyte activation (Loeb , 1952, Austin, Yanagimachi 1994). It has long been known that successful fertilization is dependent on the extracellular ionic environment, in large part because this can modify the intracellular composition of the gametes. The first observation was published 80 years ago when Loeb (1952) noted that fertilization in the sea urchin did not occur in the absence of extracellular Ca^{2+}. Later studies revealed that this was due to defective sperm function, namely failure of the acrosome reaction to occur. After the development of successful culture systems for mammalian gametes, it was possible to demonstrate that mammalian sperm fertilizing ability, like that of invertebrate sperm, can be modulated by alterations in extracellular components (Fraser , 1995).

Mammalian sperm are not immediately fertile upon release from the male reproductive tract, despite their ability to exhibit vigorous motility. They require species dependent period of time during which they undergo a series of changes, collectively referred to as "capacitation" (Austin, 1952; Yanagimachi 1994), that are needed for cells to become fully competent to fertilize an oocyte. When capacitated, mammalian sperm can (i) express hyperactivated motility, the very vigorous, thrusting pattern of motility that is needed for penetration of the oocyte investments (Yanagimachi 1994) and (ii) interact with oocytes (including cumulus cells, follicular fluid and zona pellucida) to undergo the acrosome reaction. The acrosome reaction is an exocytotic event that promotes interaction and penetration through the zona pellucida and confers fusogenic properties on the

remaining plasma membrane in the sperm head (Yanagimachi 1994). It has been suggested (Bedford 1983) that the importance of capacitation may actually be to prevent sperm from becoming fertile too quickly, given that spermatozoa are deposited into the lower regions of the female reproductive tract and must then move some considerable distance to reach the site of fertilization.

New information focussing on the mammalian sperm acrosome reaction (AR) has accumulated during the past few years, including data on (i) the mechanism of the AR (ii) the role of the AR in the fertilization process (iii) characterization of AR abnormalities influencing fertility, and (iv) methods whereby these abnormalities can be diagnosed and treated.

During a Consensus Andrology Workshop it was generally agreed that assessment of spontaneous AR was not a test of sperm function, rather than it was an assessment of sperm dysfunction; i.e. men whose spermatozoa showed a high level of spontaneous AR within a short time after separation from seminal plasma would likely have a problem of acrosomal instability. The normal situation is for spermatozoa not to show substantial spontaneous acrosome loss during capacitation, either *in vivo* or *in vitro*. *In vivo* capacitated human spermatozoa bind to ZP3 and consequently undergo the AR.

The acrosome reaction as a receptor-mediated exocytotic process

The AR is a receptor-mediated cellular response and many recent studies have investigated the nature of the receptors involved in the response and the manner in which AR-inducing signals are transduced from the receptors to the membrane fusion effectors responsible for the ensuing exocytotic reaction. *Progesterone* and a highly conserved *zona pellucida glycoprotein* termed ZP3 have been identified as natural oocyte-associated AR-inducing ligands, and their sequential action has been shown to support the occurrence of the physiological AR (Melendrez *et al.* 1994; Roldan *et al.*, 1994). There are also apparent divergences between the two pathways because the one used by the zona pellucida ligand involves a pertussis toxin-sensitive G protein (Franken et al., 1993), whereas that used by progesterone does not (Tesarik et al., 1993b). Moreover, progesterone also stimulates transmembrane chloride fluxes employing a plasma membrane channel sharing some but not all properties with the neuronal y-aminobutyric acid A ($GABA_A$) receptor (Wistrom and Meizel 1993; Blackmore *et al.*, 1994; Shi and Roldan 1995).

It is well known that progesterone opens a sperm plasma membrane calcium channel, and activates posphotyrosine kinase independently of each other (Mendoza *et al.*, 1995). Progesterone therefore reacts with a multi-receptor system on the sperm surface and this system co-operates with that used by ZP3

to control the physiological AR. Each of the respective receptors alone can eventually induce some of the AR events and in some case complete acrosomal exocytosis.

The AR must be accurately timed to ensure fertilization (Tesarik, 1989), since a premature AR leads to the loss of zona pellucida recognition sites from the sperm surface, and thus impairs sperm-zona pellucida binding (Franken et al., 1993, Liu and Baker, 1994), On the other hand inability of zona-bound spermatozoa to activate the AR also prevents zona penetration.

Acrosome reaction ionophore challenge test (ARIC)

The finding that the AR must be precisely timed with respect to sperm-zona pellucida interaction to ensure zona pellucida penetration (Tesarik, 1989) resulted in the development of the AR to ionophore challenge (ARIC) test (Cummins *et al.*, 1991). The proponents of this test have shown that it provides a more reliable picture of sperm fertilizing as compared with a test that simply measures the frequency of spontaneous AR. The practical usefulness of the ARIC test has been confirmed by other workers (Cummins, 1994).

With the use of the ARIC test two types of AR pathology have been defined***: 'AR insufficiency'*** (Tesarik and Mendoza, 1993) and ***'AR prematurity'*** (Tesarik and Mendoza 1995). AR insufficiency describes cases in which the difference in

frequency of AR between ionophore-treated and untreated aliquots of a capacitated sperm population is <15%, while AR prematurity is used for cases in which the frequency of spontaneous AR is >20% (Tesarik and Mendoza, 1995). Both pathologies can occur in the same patient. AR insufficiency as revealed by the ARIC test, can only reflect anomalies situated downstream of the calcium influx in the signal transduction cascade responsible for AR induction. At present, the most common type of test is based on the ARIC test (Cummins *et al.*, 1991).

In diagnostic laboratory practice, a physiological inducer of the AR is not used, not only because good correlations have been found between the response of human spermatozoa to calcium ionophore and their fertilizing ability, but also because human zonae are unavailable in adequate quantities for routine use. While several research groups are working on the production of recombinant human ZP3 (rhuZP3), it is not yet reliably available in a biologically active form in significant amounts. The general opinion, however, is that rhuZP3 will be the ultimate agonist or trigger substance for the human sperm AR, and will almost certainly from the basis of the 'perfect acrosome reaction test' of the future.

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CHAPTER 2

2. MATERIAL & METHODS

The material and methods used for each study is outlined in the individual articles presented in Chapter 3.

2.1 Sperm collection and analysis

The "basic" semen analysis performed by the infertility specialist included the assessment of physical semen characteristics (volume, pH agglutination, viscosity), evaluation of sperm concentration, progressive motility, normal morphology (Kruger et al., 1986, WHO 1999) and viability, presence of leucocytes and immature sperm cells, detection of antisperm antibodies and a bacteriologic investigation (Oehninger et al., 1995). A total of 338 semen samples were used during the entire study. All semen samples were collected by masturbation after 2-3 days sexual abstinence. Collections were made in sterile plastic containers at the laboratory after which the semen was allowed to liquefy for 30 minutes at 37^{O}C. Since sperm morphology formed a cornerstone in our study we prepared in all cases separate semen smears to assess the percent normal spermatozoa present in each ejaculate.

2.2 Sperm preparation

In vitro fertilization (IVF)

In cases where the couples were treated with IVF a standard single-wash swim up separation technique was used to separate motile sperm fraction from the ejaculate. The procedure is simple rapid and effective, whereby highly

progressive motile sperm are allowed to swim up from a concentrated sperm pellet into a small volume of overlying culture medium.

In short, liquefied semen was washed in culture medium ASP-100 (Scandinavian IVF Science products, Gothenburg, Sweden) for 10 minutes at 1700rpm. The sperm pellet was layered with 1.5ml IVF-50 medium (Scandinavian IVF Science Products, Gothenburg, Sweden) and incubated for 30 minutes at 37^0C and 5% CO_2 to achieve a swim-up separation of motile cells. After the incubation period, 1.0ml of the top sperm suspension was aspirated and placed in a plastic tube. Depending on the experimental design of the study a small drop or portion was removed to prepare slides for morphology and/or chromatin packaging quality and/or acrosomal responsiveness.

Intracytoplasma sperm injection (ICSI)

The metaphase II oocytes were exposed to 0,1% hyaluronidase for one minute. By aspirating the oocytes into a sterile glass drawn pipette the oocytes were stripped from the surrounding corona radiata cells. These oocytes were rinsed in fresh culture medium and prepared for ICSI.

An individual morphologically normal sperm was immobilised by perpendicular movement of the injection pipette across the tail of the sperm. The pipette fixed the tail between the to the bottom of the Petri dish for a very short time. After immobilisation the sperm was aspirated tail first into the Cook injection pipette.

Upon penetration of the zona pellucida, the ooplasm was aspirated into the injection pipette until the oolemma raptured. The sperm was deposited in the ooplasm of the oocyte as far away from the first polar body as possible. The injected oocyte was then incubated according to the IVF protocol.

Rescued intracytoplasmic sperm injection (RICSI)

The oocytes of patients where no fertilization was reported one day after aspiration, were subjected to RICSI. Reinsemination by ICSI was performed by mechanical introduction of a spermatozoon into the oocyte. In short, oocytes were scored for the presence of a polar body and incubated in 100ul culture medium under mineral oil in Falcon 1006 Petri dishes. Spermatozoa for ICSI were put in a droplet of polyvinylpyrrolidone (PVP) and aspirated into the injection pipette. The sperm was immobilized by imposing pressure (using the injection pipette) on the tail of the sperm. A single spermatozoon was injected into the ooplasm of the oocyte once the oolemma was broken.

2.3 Preparation of solubilized zona pellucida

In all the experiments where solubilized zona pellucidae were needed, unfertilized oocytes (no pronuclei or second polar body) donated by diagnosed male factor couples from the in vitro fertilization program were used. Oocytes with <10 spermatozoa attached to the zona pellucida were rinsed in Dulbecco's Phosphate Buffered Saline (DPBS, D-5773, Sigma Aldrich, Midrand. Republic of South Africa). Twenty oocytes were placed in 0.1ml DPBS in an Eppendorf 0.5ml

micro tube (Micro Tubes, Safe-Lock, Eppendorf-Netheler-Hinz-GmbH, Hamburg, Germany) at 4^0C. The oocytes were washed by centrifugation for 10 minutes at 2600rpm. Using a stereoscope, DPBS was then gently aspirated with a glass drawn Pasteur micropipette. Acid Tyrode's (T-1788, Tyrode's Solution Acidic, Embryo tested, Sigma Aldrich, Vorna Valley, Republic of South Africa) was added and oocytes were centrifuged again. . The Tyrode's solution was gently aspirated once more prior to the addition of 20µl fresh Acid Tyrode's. The zonae were dissolved at room temperature (45-60 min) in Acid Tyrode's by agitating the -zonae solution every 5 minutes with a glass drawn Pasteur Pipette. The reaction was neutralised, by adding 20µl 1mM NaOH. Zonae solutions with a concentration of 0.5 zona pellucida per microlitre (0.5ZP/µl) were stored up to two weeks at 4^0C.

2.4 Chromatin packaging quality

During the preliminary experiments, technician and sample variation was recorded, by establishing inter assay coefficient of variation (CV) for CMA_3 staining. Intra-assay variation was determined by evaluating 100 cells on ten different microscopic fields (total 1000 cells) from the same semen specimen. Inter assay variation was accomplished by evaluating staining for CMA_3 by counting 200 cells on 5 different specimens from the same sperm donor. Coefficient of variation (Mortimer, 1994) for both intra-and inter assay values were calculated using the following formula:

$$CV\ (\%) = \frac{\text{standard deviation}}{\text{mean}} \times \frac{100}{1}$$

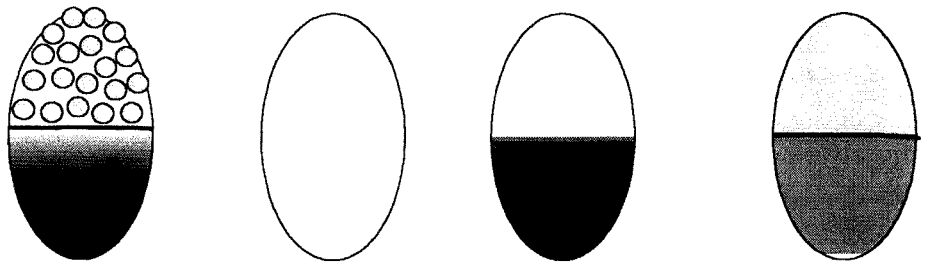
The CV during the standardization of results were <12% in all instances.

2.4 Induction of the Acrosome reaction

2.4.1 Assessment of the acrosome reaction

The following staining patterns were evaluated as acrosome reacted spermatozoa; (i) patchy staining on acrosomal region, (ii) distinct staining in the equatorial region occurring as an equatorial bar and (iii) and no staining observed over entire sperm surface. Since we used only swim up sperm samples, the motility was in all cases >80%. Acrosome reaction data presents the findings recorded for live sperm. A minimum of 100 spermatozoa was counted and the staining patterns are depicted in Figure 1.

Figure 1
Patterns recorded during FITC-PSA staining.



Patchy Equatorial Reacted Unreacted

2.7 Oocyte evaluation

Metaphase I and II oocytes were identified according to their nuclear maturity status. In metaphase II oocytes a first polar body was present while in

metaphase I oocytes no polar body was observed. Following oocyte retrieval, the embryologist meticulously carried out the evaluation and classification of the oocytes. Under microscopic vision, metaphase II oocytes were characterized by their round, even shapes and presence of light colour and homogeneous granularity. They were always associated with expanded, luteinized cumulus and "sun-burst" corona radiata. The membranes of the granulosa cells harvested along with the metaphase II oocytes were also luteinized, loosely aggregated and had mature features (Figure 2, Veeck, 1988).

Figure 2

Metaphase I human oocyte with no polar body.

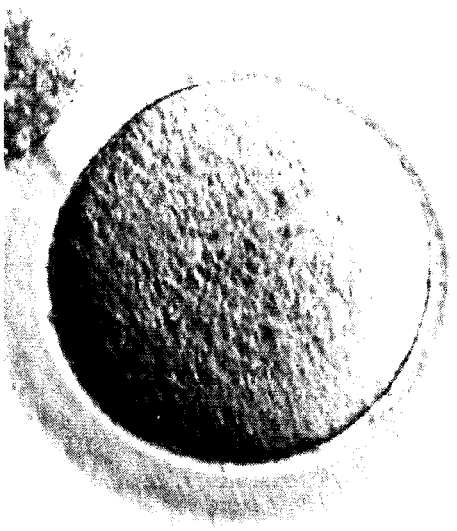
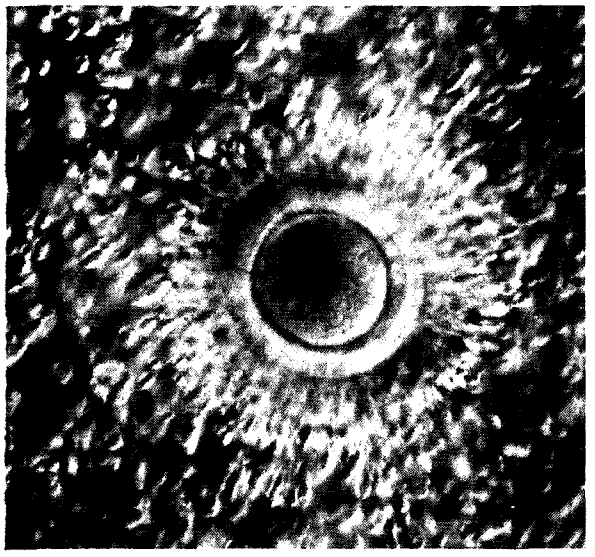
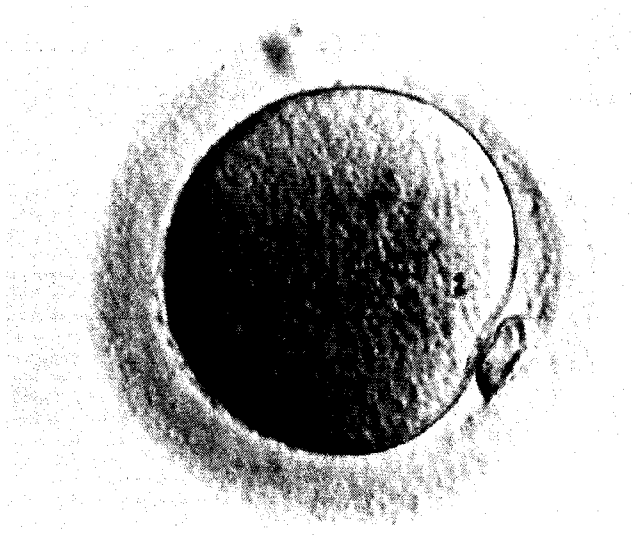


Figure 3
Metaphase II human oocyte associated with an expanded, luteinized cumulus and a "sun-burst" corona radiata and polar body





2.5 Ovulation induction

The ovulation induction protocols used for the 338 women in the study were standardized and are described in detail in Chapter 3 under the specific section of each experiment.

2.7 Statistical Analyses

The diagnostic accuracy of the specific measurement i.e. chromatin packaging quality or acrosome reaction results were illustrated with interactive dot diagrams and the positive and negative predictive values were recorded with the Receiver Operating Characteristics (ROC curve) (Aitman and Bland, 1994, Schoonjans et

al., 1995). Logistic regression was employed to model decondensation in terms of CMA_3 and morphology, where decondensation was binary (success or failure). Positive and negative predictive values were calculated with chi-square test. Group comparisons for the different parameters were performed with Fishers' exact t-test, chi -square test and Welch T-test where applicable.

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CHAPTER 3

EXPERIMENTAL DESIGN

The study included 338 couples consulting for infertility treatment at various gynaecologists in Pretoria and Johannesburg, while the diagnostic assisted reproductive laboratory support was provided by the Andrology laboratory of Drs du Buisson and partners from Pretoria.

The experimental design has been divided into five sections that are presented as separate research papers. The articles each have a specific summary, introduction, material and methods, results, discussion and reference sections pertaining to the study concerned. Although each section of this chapter may be regarded as a complete and separate experiment, the order of presentation adheres to the sequential investigation previously mentioned. Furthermore despite the fact that these studies are discussed as separate entities in this chapter, it is important to remember that they remain closely related, since they all have a mutual goal i.e. to further our understanding of the human fertilization process.

Sperm chromatin packaging as an indicator of in vitro fertilisation rates

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Abstract

The development of a sequential diagnostic schedule for patients consulting for infertility problems would be an ideal method of approach for clinicians in the absence of an etiological or pathophysiologic diagnosis. Since sperm morphology recorded by strict criteria, has often been correlated with fertilization failure, the present study aimed to evaluate the relationship between normal morphology as well as IVF rates with chromatin staining among fertile and subfertile men. Two semen smears were prepared from each specimen obtained from 72 men to record normal morphology and chromatin packaging as recorded by CMA_3. Following the semen analyses, the 72 men were divided into the 2 morphologic groups, namely, <4% and >4% normal forms. Significant differently percentages CMA_3 staining (mean±SE) were recorded among the 2 morphology groups, namely 65.9%±3.5 and 44.5%±1.7 (p=0.001).

A negative and highly significant correlation existed between percentage normal morphology as recorded by strict criteria and CMA_3 staining. A significant and

positive correlation was recorded for normal morphology and in vitro fertilisation rates (r = 0.45, p=0.0001). A negative and significant correlation (r = 0.51, p=0.0001) existed between CMA_3 values and in vitro fertilisation results. The discriminating power of nuclear maturity, as recorded by CMA_3 staining, to identify abnormal morphology values and poor in vitro fertilisation, were calculated with Receiver Operator Characteristics (ROC) analyses. The areas under the ROC curve were 0.86, for sperm morphology and 0.74 for in vitro fertilisation. The calculated cut off value for CMA_3 staining to distinguish between morphology groups, was 48% and 50% for IVF. Chromatin packaging assessment is a valuable addition to the sequential diagnostic programme in an assisted reproductive arena.

Introduction

Semen quality is traditionally determined according to sperm concentration, motility and morphology features in a given ejaculate (World Health Organization, 1987). Likewise, it is accepted that an association exists between sperm morphology as recorded by strict criteria and in vitro fertilization rates (Kruger et al., 1986, Liu et al 1988, Oehninger et al., 1988). Sperm morphology is also related to the incidence of other sperm deficiencies, including poor zona pellucida binding (Franken et al., 1996) and penetration (Lanzendorf et al., 1994), a poor response to agonists that modulate intracellular calcium concentrations (Oehninger et al 1994) and a high content of creatine kinase (Huszar et al., 1994).

Poor chromatin packaging as indicated by elevated chromomycin A3 staining values (>40%) and possible DNA damage, may contribute to failure of sperm decondensation after ICSI and subsequently result in fertilization failure (Bianchi et al., 1996, Sakkas et al., 1995, Sakkas et al., 1996, Lopes et al., 1998).

Several techniques has been described such as the Sperm Chromatin Structure Assay (SCSA) which uses the metachromatic properties of acridine orange, other dyes include the use of aniline blue (Henkel et al., 1994) and fluorochromes, for example, methyl green (Godowicz 1977), giemsa stain (Windt et al., 1994), ethidium bromide (Filatov et al., 1999), acridine orange (Evenson et al., 1999), and chromomycin A_3 (Bianchi et al., 1996) (CMA_3). Chromatin of mature sperm has been shown to possess a varying binding capacity for many of the mentioned dyes and stains. The binding capacity is believed to reflect anomalies in the packing quality due the modifications of the nucleoprotein components occurring during spermiogenesis. Basically binding capacity involves replacement of histones by protamines and then further stabilisation by the formation of intra-inter-molecular disulphide cross-links among the cysteine residues of the protamine molecule (Balhorn 1989). One of these fluorochromes CMA_3 has been found to be a useful tool for the detection of both protamine-deficient loosely packaged chromatin and nicked DNA (Manicardi et al., 1995). The strong correlation that has been shown to exist between sensitivity to CMA_3 staining and sensitivity to endogenous in situ nicks translation has been reported by various groups (Manicardi et al., 1995). Sperm chromatin integrity as reflected

in a ratio of single to double stranded DNA, as measured by the SCSA, is predictive of infertility and subfertility in a range of species including humans (Bizzaro et al., 1998, Evenson et al., 1986, Evenson et al., 1994).

The present study aimed to evaluate the relationship between sperm morphology and the recorded IVF rates, with chromatin staining, among men with >4% and <4% normal spermatozoa.

Material & Methods

Sperm preparation

Semen samples from 72 men were collected on the day of the IVF treatment of their wives. Samples were analyzed for sperm concentration, % motility and forward progression using World Health Organization guidelines (WHO, 1987). Sperm morphology was evaluated by strict criteria (Kruger et al., 1986). Two semen smears were prepared from each specimen to record normal morphology and chromatin packaging as recorded by CMA_3 (Bianchi et al., 1996) staining. Samples were classified as fertile and sub-fertile according to the percentage normal spermatozoa (Kruger et al. 1986). Separation of the motile fraction was accomplished following a double wash swim up method using Ham's F10 supplemented with 10% human foetal cord serum.

CMA_3 staining

Semen smears were fixed in methanol/glacial acetic acid 3:1 at 4^0C, for 20 minutes. Slides were allowed to air dry at room temperature for 20 minutes. For CMA_3 staining (Sigma Chemicals, St Louis, MO USA Cat 2659), each slide was treated for 20 minutes with 100 µL CMA_3 solution. The CMA_3 solution contained of 0.25mg/mL CMA_3 in McIlvane's buffer (Geigy Scientific Tables 1984), pH 7.0, supplemented with 10mM $MgCl_2$..

Slides were rinsed in buffer and mounted with Dabco (Aldrich Chemicals Co, Milwaukee, USA cat No. 29,073-4). The slides were then kept at 4^0C overnight after which evaluation of fluorescence was performed the following morning using a Nikon Labophot 2 (CFWN10X IMP., Johannesburg South Africa) fitted with a triple band filter-FITC, rhodamine, DAP1 and an Eplan 100X objective. The lens allowed the use of both phase and fluorescence. A total of 200 spermatozoa were randomly evaluated on each slide.

Evaluation of CMA_3 staining was done by distinguishing between bright yellow stained sperm (CMA_3 positive) and dull yellow stained sperm (CMA_3 negative). A clear distinction existed between CMA_3 positive and negative sperm, since CMA_3 positive sperm revealed an intensive bright fluorescent yellow appearance. Prior to the onset of the study, intra- and inter assay variations were recorded for fertile sperm donors.

Standardisation of results

During the preliminary experiments, technician and sample variation was recorded, by establishing inter assay coefficient of variation (CV) for CMA_3 staining. Intra-assay variation was determined by evaluating 100 cells on ten different microscopic fields (total 1000 cells) from the same semen specimen. Inter assay variation was accomplished by evaluating staining for CMA_3 by counting 200 cells on 5 different specimens from the same sperm donor. Coefficient of variation (Mortimer, 1994) for both intra-and inter assay values were calculated using the following formula:

$$CV\ (\%) = \frac{\text{standard deviation}}{\text{mean}} \times \frac{100}{1}$$

The CV during the standardization of results were <12% in all instances.

In vitro fertilisation

Ovulation induction was achieved using a combination of chlomiphene citrate (CC. Serophene, Serono, Rome, Italy) 100mg daily on days 4 through 8 of the woman's menstrual cycle, as well as human menopausal gonadotrophin (HMG-Pergonal, Serono, Rome, Italy). The dosage of HMG. was administered according to follicular response as assessed by transvaginal ultrasound and daily serum oestradiol. The stimulation program is individualised according to the patient's response during previous cycles. In some cases stimulation was

accomplished using a combination of gonadotrophin releasing hormone agonist (GnRH-a), Buserelin nasal spray (Suprefact, Hoechst AG, Frankfurt, Germany) and HMG., HCG was given (10 000IU, Pregnyl, Organon, Istanbul, Turkey) when two or more follicles reached or exceeded a mean diameter of 18mm with serum oetradiol concentrations of 1800pmol.

Follicular aspiration for oocyte collection was performed 36 hours following the administration of HCG. Ham's F-10 (Gibco BRL, Life technologies Ltd., Paisley, Scotland) supplemented with 10% human foetal cord serum was used for insemination and culture medium. Follicular fluid was aspirated in sterile tubes (Falcon Plastics Cat 2001, CA, USA). The maturational stage of the oocytes was microscopically assessed after which the oocytes were rinsed in small Petri dishes (Falcon Plastics Cat 3001 CA, USA) containing 4.0 ml Ham's F10 supplemented with 10% human foetal cord serum. Incubation of the gametes as well as the culturing of embryos took place at 37°C, 5% CO_2 in a center well Petri dish (Falcon Plastics 3037, Cat 3001 CA, USA). No more than three oocytes were incubated in a single Petri dish. Ham's F10 supplemented with human foetal cord serum was used as culture medium. The human foetal cord serum was filtered, inactivated and tested for the infectious diseases i.e. HIV and Hepatitis.

Oocytes were inseminated with a 100 000 - 500 000 motile spermatozoa per ml in accordance to the percentage morphological normal forms present. Corona

and cumulus cells were removed 16-18 hours after insemination and fertilization was confirmed when 2 pronuclei were identified. Culturing proceed until the embryos reached a six to eight cell stage. Three to four embryos were transferred on day three using a Tomcat catheter. CMA_3 staining was performed on all semen samples obtained from the 72 men on the day of the procedure, stained and read the following day.

Statistical analysis

Comparisons between normal sperm morphology and in vitro fertilisation percentage chromatin staining were done using correlation analysis. The discriminating power of chromatin packaging as a screening test for the identification of normal sperm morphology and in vitro fertilisation was illustrated with Receiver Operating Characteristics (ROC) (Altman and Bland, 1994).

Results

Normal sperm morphology (strict criteria)

Following the semen analyses, the results of the 72 men were divided into the 2 morphologic groups, namely, <4% and >4% normal forms. Significantly lower percentages of CMA_3 staining (mean±SE) were recorded between the 2

morphology groups, namely 65.9%±3.5 and 44.5%±1.7 (p=0.001), respectively (Table 1).

Table1

Semen characteristics (mean ±SE) and in vitro fertilisation results of 72 patients with CMA3 staining in sperm morphology groups >4 and ≤4% normal spermatozoa

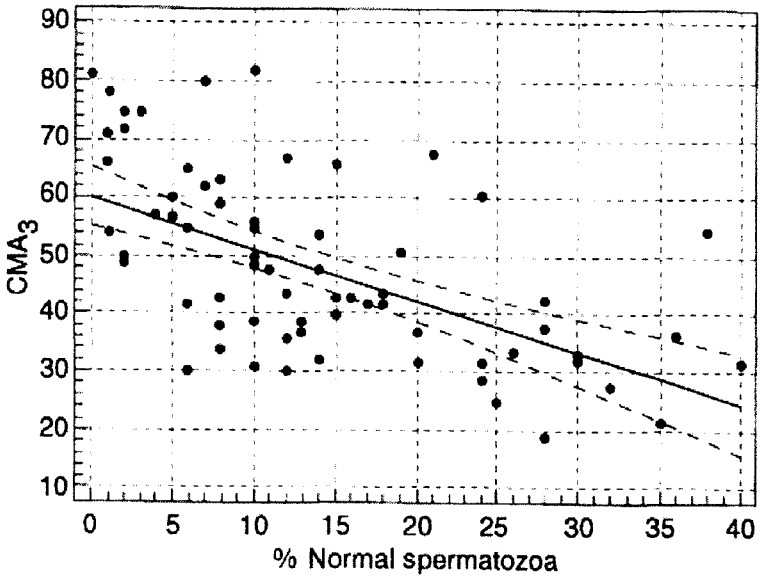
% normal forms	% CMA3 staining	% normal forms	Sperm conc. 10^6/ml	% motile	# oocytes	% oocytes fertilised
≤4%	65.9	1.4	22.5	60.0	6.2	14.9
n=11	±3.5a	±0.2c	±7.3e	±3.0	±1.4g	±5.5i
>4%	44.5	16.2	62.5	61.7	6.9	57.3
n=61	±1.7b	±1.1d	±4.0f	±1.7	±1.2h	±3.7j

Unpaired student's t-test: a vs. b: $p<0.01$, c vs d; $p<0.01$, e vs f; $p<0.01$, g vs h: $p>0.05$, i vs j: $p<0.01$

A negative and highly significant correlation existed between percentage normal morphology as recorded by strict criteria and CMA_3 staining (Figure 1, $r = -0.59$, $p=0.0001$).

Figure 1

Scatter diagram of CMA_3 staining and percentage normal sperm morphology of 72 men. The solid line indicates the regression line representing the relationship between CMA_3 staining and the percentage of morphologically normal sperm in raw semen. Dashed lines indicate the 95% confidence interval.

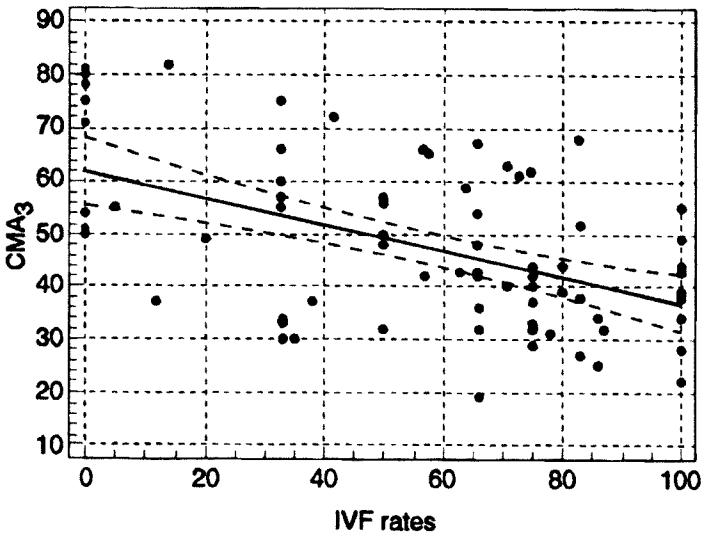


In agreement with previous reports a significant and positive correlation were recorded for normal morphology and in vitro fertilisation rates ($r = 0.45$, $p = 0.0001$).

A negative and significant correlation (Figure 2, r =-0.51, p=0.0001) existed between CMA_3 values and in vitro fertilisation results.

Figure 2

Scatter diagram of CMA3 staining in vitro fertilisation rates. The solid line indicates the regression line representing the relationship between CMA_3 staining and in vitro fertilization rates. The dashed lines indicate the 95% confidence interval.



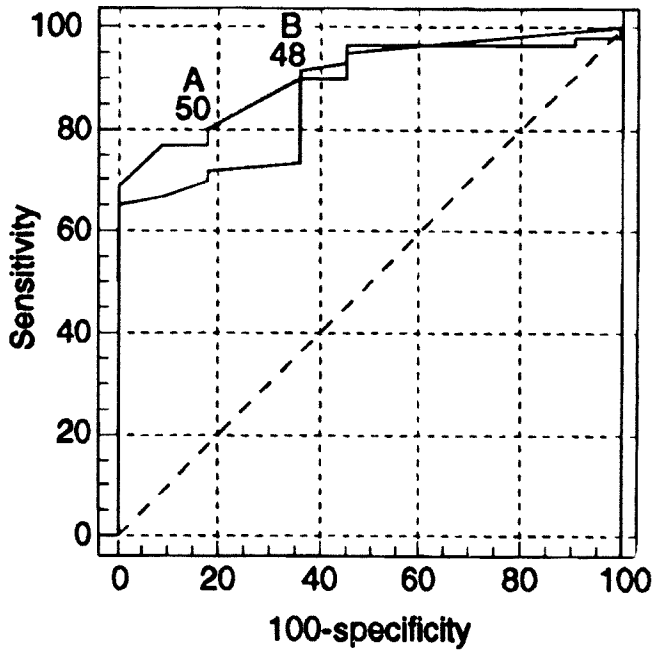
The mean number of oocytes retrieved among the <4% and >4% morphology groups, were 6.2±1.4 and 6.9±1.2, respectively (p>0.05). The mean percentage oocytes fertilised among the 2 morphology groups were 14.9±5.5% (<4% normal morphology) compared to 57.3±3.7% (>4% normal morphology). Fertilisation rate was defined as the number of oocytes fertilised when the extrusion of the second polar body was observed 18 hours after insemination.

The discriminating power of nuclear maturity, as recorded by CMA_3 staining, to identify abnormal morphology values and in vitro fertilisation rates with <60% of

metaphase II oocytes fertilized, were calculated with Receiver Operator Characteristics (ROC) analyses. During ROC analyses (Figure 3), we used varying sperm morphology values to calculate the optimum sensitivity and specificity levels for CMA_3 staining and in vitro fertilisation rates. The areas under the ROC curve were 0.74 for in vitro fertilisation and 0.86 for CMA_3 staining. The calculated cut off value for CMA_3 staining, to distinguish between the selected morphology groups, was 48%. When in vitro fertilisation rates were used in the ROC analysis, the CMA_3 cut off value for accurate prediction was 50%.

Figure 3

Receiver Operator Characteristics analyses of CMA_3 staining, percentage normal sperm morphology and in vitro fertilisation rates. Optimal prediction of IVF occurred at CMA_3 values of >50% (A), while morphology group prediction occurred at CMA_3 values of >48% (B).



Discussion

The successful implementation of intracellular sperm injection (ICSI) has provided a unique means to allow couples suffering from severe male infertility to achieve their reproductive goals. However, despite the great therapeutic advantages of the technique, ICSI provides solutions to clinicians often in the absence of an etiological or pathophysiologic diagnosis. In the face of this new technique, several questions obviously arise including (i) what are the diagnostic steps that we should use to direct infertile men to a specific therapeutic modality? and (ii) what are the current indications for ICSI?

The andrologic investigation still relies on a thorough history and physical examination of the male partner. The semen analysis still remains the cornerstone of the diagnostic management. We have been promoters of a sequential, multi-step diagnostic approach for the evaluation of the various structural, dynamic and functional sperm characteristics (Oehninger et al., 1991).

The power of the morphology of a given sperm sample (>4% and <4% normal forms) to predict the CMA_3 staining quality of the sample, was reflected in Figure 3 by the area under the ROC curve, namely 86%, while the area under the curve for in vitro fertilisation was 74% for the selected morphology groups. An area under the curve of 0.86 implies that a randomly selected individual with CMA_3 staining of >48%, will have increased CMA_3 staining in 86% of cases. The

confidence interval (0.8 to 0.96) does not include 0.5, implying that CMA_3 staining has the ability to distinguish between morphology groups >4% and <4%. When CMA_3 staining is used as a discriminator of sperm morphology, the sensitivity, that is the probability that the percentage CMA_3 staining will be elevated (>48%) in the presence of poor sperm morphology, was calculated at 65%. The specificity, that is the probability that CMA_3 staining will reflect decreased staining values (<48%) among the sperm morphology group >4% normal forms, was 100%.

When CMA_3 staining is used as a discriminator of in vitro fertilisation success (>50% oocytes fertilised) a sensitivity of 73% and specificity of 75% was recorded. The 95% confidence interval did not include 0.5 (0.60 to 0.83), meaning that CMA_3 staining could distinguish between IVF success and failure. An area under the curve of 0.90 means that a randomly selected individual with CMA_3 staining of >50%, will fertilise >50% of metaphase II oocytes in 90% of the cases.

It is well documented that selection of motile sperm by swim up can improve the quality of the subfertile sperm recovered, with respect to morphology (Menkveld et al., 1991), motility (Ng et al 1992) and nuclear maturity (Le Lannou and Blanchard, 1988). Several workers in the field of assisted reproduction, documented the clinical relevance of strict sperm morphology i.e. significant lower fertilization rates reported during in vitro fertilization treatment (Oehninger et al., 1991) as well as impaired sperm-mucus interaction and reduced

fertilisation capacity under natural conditions of conception (Eggert-Kruse et al., 1995).

Although it is evident that sperm in semen containing a high level of abnormal forms, has a reduced fertilising potential, the true anomalies present in abnormal sperm cells have only partially been characterised. Specific biochemical markers have been associated with abnormal sperm, possibly the most important being the association with reactive oxygen species production (Aitken and Clarkson, 1988) and the enhanced creatine phosphokinase activity present in abnormal spermatozoa (Huszar et al., 1994). The present data are in close agreement with the conclusions of other studies (Bianchi et al., 1996), that poor sperm exhibiting abnormal morphology are more likely to possess loosely packaged chromatin. It appears that abnormal sperm morphology is an overall indicator of sperm that have failed to progress through spermiogenesis. As a consequence they may display characteristics typical of immature spermatozoa, which is also evident as previously stated, in the biochemical composition (Huszar et al., 1994).

Even though good sperm morphology data have been correlated with fertility success, a normal shaped sperm head may contain chromosomes with microdeletions, aneuploidy, DNA strand breaks, and abnormal sperm chromatin structure and yet fertilize an oocyte (Evenson et al., 1999). Spano et al. (1998) used the SCSA and illustrated that the results can be influenced by the age of

the sperm donor, smoking habits, the presence leukocytes and immature germ cells in the ejaculate and the duration of sexual abstinence. On the other hand, the relationship between SCSA results and sperm concentration, morphology and vitality was weak ($-0.22<r<-0.46$).

During a recent study (Sakkas et al., 1996) patients with increased CMA_3 staining (>40%) also had more condensed spermatozoa in unfertilised ICSI oocytes as compared to oocytes from patients with lower CMA_3 staining (<30%). The percentage of sperm which had the capacity to initiate decondensation in unfertilised oocytes was not influenced by morphology or CMA_3 staining (Sakkas et al., 1996). Lopes et al. (1998) also showed that DNA damage in sperm might contribute to fertilisation failure after ICSI. Flow cytometric analysis of sperm DNA randomly chosen from 100 men undergoing fertility treatment revealed a general association between impaired sperm quality, as recorded by conventional characteristics, and the appearance of sperm with poor chromatin condensation in the ejaculate as expressed by DNA fluorescence intensity (Evenson et al 1999).

Chromatin hypocondensation, depicted by increased fluorescence, was present in different degrees in different sperm samples (Engh et al., 1992). It is therefore logical to assume that poor chromatin packing as indicated by an increase percentage sperm showing intensive CMA_3 staining, may contribute to failure of sperm decondensation after ICSI and subsequently result in fertilization failure.

Although we do not postulate that fertilization is due to a single sperm defect, it seems likely that poor chromatin packaging may contribute to a failure in the decondensation process. Auger et al. (1990) used acidic aniline blue staining to detect chromatin defects of sperm nuclei related to nucleoprotein content as associated with DNA.

Semen characteristics that discriminated significantly between fertile and possible infertile men were (1) the percentage of normal sperm, semen volume and the percentage of mature sperm heads. These results indicate that the addition of the evaluation of sperm head maturity to routine semen analysis improves the assessment of fertility in men.

On the other hand, the packaging of the DNA within the human sperm nucleus appears to be unique. The nucleohistone fraction and nucleoprotamine fractions of mature sperm chromatin seem to have sequence-specific relationship. The complex chromatin structure imposed by alternating regions of nucleosome and protamine bound sperm DNA and the presence of RNA demonstrate that the spermatozoon genome might be in a semi-active muted state prior to fertilization. Many of the late post-meiotically transcribed genes are synthesized during the final stages of transcription prior to the selective transition protein mediated replacement of histones by protamines (Kramer and Krawetz 1997).

The fate of the individual mRNAs and the function of their corresponding potentiated genic domains during fertilization and early development is unknown. Kramer and Krawetz (1997) suggested the possibility whether these mRNAs present in mature sperm, may serve some yet unidentified role in post fertilization events within the one cell embryo. Little is known with regard to the mechanisms by which the protamines are replaced during the decondensation of the paternal complement in the fertilized zygote.

Since ICSI overrides deficiencies in sperm motility, zona and oolemma binding and leaves the successful completion of fertilisation upon the sperm nucleus, we suggest that chromatin packaging assessments should be included as a complementary assay to the sequential diagnostic approach of the male factor patients. The evaluation of DNA status in cases where repeated centrifugation of sperm was used to prepare the sperm, is indicated, since a significant increase in reactive oxygen levels was reported. The ROS levls correlated significantly with DNA damage. (Twigg et al. (1998).

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Chromatin packaging as an indicator of human sperm dysfunction

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Abstract

Purpose: The study aimed to evaluate the relationship between chromatin packaging quality and (i) normal morphology and (ii) its ability to predict the functional integrity of spermatozoa during IVF and ICSI treatment.

Methods: Semen of 140 men from IVF and ICSI couples were analyzed for sperm concentration, motility, morphology and chromatin packaging (CMA_3). IVF and ICSI data were stratified using 3 basic cut off values for CMA_3 staining, namely <44%, >44-60% and >60%.

Results: CMA_3 staining results divided the results four CMA_3 groups, namely; Group A (IVF); CMA_3<44%; Group B (IVF); CMA_3≥44% and <60%, Group C: CMA_3≥60%(IVF); Group D; CMA_3≥60%(ICSI). Receiver Operator Characteristic analyses calculated the cut off value for CMA_3 to distinguish between morphology <4% and ≥4%, was 60%. `When IVF rates of >60% and <60% were used the optimal CMA_3 value for prediction of fertilization success was recorded at 60%.

Conclusions: Chromatin packaging assessments should be included as a complimentary assay to the sequential diagnostic approach of the male factor patients.

INTRODUCTION

Sperm dysfunction is one of the most common single causes of male infertility yet, remarkably, our knowledge of the cellular and biochemical basis for this condition is very limited. Indeed, our understanding of the physiology of the normal human spermatozoon, let alone the dysfunctional spermatozoon, is elementary. The successful implementation of ICSI has provided a unique means to allow couples diagnosed with severe male infertility to achieve their reproductive goal (1). However, despite the great therapeutic advantages of the techniques, in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) provides solutions to clinicians often in the absence of an etiological or pathophysiologic diagnosis.

The underlying cause for their infertility is generally related to specific sperm defects that generally comprise of low counts ($<1x10^6$/ml) and/or low motility (<30%) and/or severe teratozoospermic men (2). Patients with these semen parameters are usually directly referred to the ICSI programme. ICSI has recently also been applied to patients with obstructive azoospermia, since ICSI is also successfully applied using epididymal and testicular spermatozoa (3, 4).

Current important issues being investigated among couples attending assisted reproductive programmes include the determination of chromosomal abnormalities and sperm functional quality (5). The need to employ a multi-diagnostic approach during the clinical workup has been underlined by the participants of a Consensus Workshop in Advanced Andrology and it suggested that sperm functional assays should become part of the male diagnostic programme (6,7,8).

Sperm chromatin packaging as an indicator for fertilization in assisted reproduction has recently been underlined (9,10,11). To evaluate the chromatin of mammalian sperm, numerous dyes and fluorochromes have been used, for example, aniline blue, methyl green, giemsa stain, ethidium bromide, acridine orange and chromomycin A3 (12,13). The binding capacity of these dyes is believed to reflect anomalies in the chromatin packing quality due the modifications of the nucleoprotein components occurring during spermiogenesis. Basically it involves replacement of histones by protamines and then further stabilisation by the formation of intra-inter-molecular disulphide cross-links among the cysteine residues of the protamine molecule (13).

The aim of this study was to evaluate the possible relationship between CMA3 staining and (i) normal morphology and (ii) its ability to predict the functional integrity of spermatozoa in both IVF and ICSI treatment programmes.

MATERIAL AND METHODS

Experimental design

Based on semen parameters, 140 consecutive patients were directed to either the IVF or ICSI programme. Patients with <4% normal forms, $<1x10^6$ sperm/ml and ≤40% motile cells (except for one patient with a motility of 50%) and a history of repeated failed IVF, were admitted to the ICSI programme Patients in the IVF and ICSI programme, were classified according to CMA_3 staining. The criteria for CMA_3 classification were based on the results obtained during a previous study, where 44.5%±13 was reported to be a cut off value for CMA_3 staining among fertile and sub-fertile men (14). This value was similar to that reported by others (11,12). In order to analyse the data of the present study; we calculated a second cut off value estimated at 60.0% recorded as 1xSD above 44.5%. Accordingly the data were stratified using 3 basic cut off values for CMA_3 staining, namely <44%, >44-60% and >60%. Data were analyzed according to CMA_3 staining values of 44.5% and 60% as cut off values. Each CMA_3 category of patients were then further divided into 2 subgroups, namely <4% normal forms and ≥4% normal forms.

Ovulation-Induction

Ovulation-induction protocols were adapted to individual needs. A "long" and "short" protocol was the most commonly used. In the “long” protocol Buserelin (Suprefact, Hoechst, IHD, Johannesburg SA), Lucrin (GnRHa, Abbott, IHD, Johannesburg, SA), and Gonal-F (recombinant FSH, Serono, Pty (Ltd),

Johannesburg, SA) were used. The "long" protocol treatment was induced in the mid-luteal or late luteal phase and the "short" protocol administration of GnRHa begins in the early follicular phase. Both treatments continued until the day of hCG (Profasi, Serono, Pty (Ltd), Johannesburg, SA) administration. FSH (Metrodin, Serono, Pty (Ltd), Johannesburg, SA) treatment was started after desensitization was achieved. The dose of FSH was tailored to the patient's response. When the largest follicle reached a diameter of 18mm and the estradiol serum levels indicated a satisfactory follicular response, 10 000 IU hCG was administered.

In vitro fertilization

Metaphase II oocytes and embryos were cultured as suggested in the IVF Science and Medi-Cult literature (15). Shortly, culture dishes (Falcon 1006, Becton Dickinson GmbH, Heidelberg) were prepared using 24 hours pre-equilibrated culture media (IVF-50; Scandinavian IVF Science products, Gothenburg, Sweden; Universal IVF medium; Medi-Cult, MØllehaven, Jyllinge) and Sigma mineral oil (Sigma-M-8410, embryo tested, Sigma-Aldrich, Johannesburg, SA). Three-ml IVF-50 or Universal IVF medium was incubated in Falcon 2058 tubes (Becton Dickinson GmbH, Heidelberg) for the final swim-up or suspension of spermatozoa after employing a density gradient column separation. Spermatozoa were washed in ASP-100 (Scandinavian IVF Science products, Gothenburg, Sweden); or Flushing Medium (Medi-Cult, MØllehaven,

Jyllinge) and layered with 1,5ml IVF-50 or Universal IVF. Insemination and oocyte culturing took place in IVF-50 or Universal IVF medium. On day one oocytes were denuded and fertilization assessed. Zygotes were transferred into six 50ul droplets IVF-50 (Scandinavian IVF Science products, Gothenburg, Sweden) or Universal IVF (Medi-Cult, MØllehaven, Jyllinge).

Two to four cell embryos were assessed on day two and transferred to prepared dishes, containing six 50ul droplets IVF-50 or Universal IVF medium. Culturing of day 3 embryos (8-10 cells) and embryo transfer took place in G2.2 (Scandinavian IVF Science products, Gothenburg, Sweden) or M3 Medium (Medi-Cult, MØllehaven, Jyllinge).

The quality of the embryos was evaluated according to the following system, namely Grade I, II, III and IV. Grade I embryos were those embryos where the blastomeres were equal in size and with no fragments. Whereas Grade II embryos contained minor fragmentations, with equal sized blastomeres. When fragments consisted more than one-third, but less than two-thirds of the volume of the embryo and/or blastomeres were of different sizes, the embryos were graded as Grade III. Grade IV embryos showed a high degree of fragmentation (more than two-thirds of the volume of the embryo) and blastomere unequal in size.

Intra cytoplasmic sperm injection (ICSI)

After retrieval the oocytes were incubated in 0.1% hyaluronidase for one to two minutes. The oocytes were rinsed (5 times) in fresh HEPES buffered medium. Prior to injection the oocytes (only Metaphase II) were placed in 10µl HEPES buffered droplets under oil using 1006 Falcon Petri – dish. A small volume (2-3µl) of prepared spermatozoa was deposited in a droplet of Polyvinylpyrrolidone (PVP, Cat No 10890001, Medicult, Harrilabs, Randburg, SA). Narishige Micromanipulators mounted on a Nikon Inverted Microscope were used to perform the sperm injection. An individual morphologically normal sperm was immobilized and sucked into a Cook injection pipette (KMPIP-1035). The sperm was injected and the injected oocyte was then incubated according to the IVF protocol.

Semen preparation

A total of 140 semen samples were obtained from men attending our fertility program. After complete liquefaction at room temperature, a basic semen analysis was performed according to the WHO (16, 2) method. In short recordings were made of the semen volume, concentration spermatozoa motility and forward progression. Two separate slides were prepared to evaluate the percentage normal cells and the quality chromatin packaging.

For morphology evaluation all slides were stained with Papanicolaou stain, coverslipped and then assessed for percentage normal cells according to strict criteria (2). The quality chromatin packaging was determined using CMA_3 staining (Sigma Chemicals, St Louis, MO USA Cat 2659), techniques (11,12). In short, CMA_3 staining thin smears were prepared, air dried and fixed in methanol/acetic acid 3:1 at room temperature for 20 minutes. The slides were air dried and stained with 60-100ul CMA_3 in a dark chamber for 20 minutes. Slides were then washed in McIlvaine buffer (17) and mounted using Dabco (Aldrich Chemicals Co, Milwaukee, USA cat No. 29,073-4). Two hundred spermatozoa were evaluated under a Nikon fluorescent microscope (CFWN10X IMP., Johannesburg South Africa; Filter Fx 465-495).

Statistical Analyses

The primary statistical methods employed in the studies included Spearman's rank order correlation between IVF- and ICSI groups and semen parameters, logistic regression of fertilization defined as "good' or "poor" at some cut-off value (i.e., 60%) calculated by using fertilization rates from previous IVF cycles. In addition, predictive statistics i.e. sensitivity, specificity and positive predictive value (PPV) and negative predictive value (NPV) for CMA_3 staining and IVF and ICSI results are reported. Predictive values were determined with a 2x2 contingency table while the statistical relevance was calculated with Yates

corrected chi-square test. The discriminating power of sperm morphology and in vitro fertilization as a screening test for the identification of chromatin packaging was illustrated with Receiver Operating Characteristics (ROC) (18).

Results

Patients

Based on this CMA3 classification patients were divided into 4 groups, namely; Group A; <44% CMA_3 (n=15, IVF); Group B; ≥44% and <60% CMA_3 (n=39 IVF); Group C: ≥60% CMA_3 (n=45 IVF); Group D; ≥60% CMA3 (n=41 ICSI). Ninety nine patients were admitted to the IVF programme, while 41 received ICSI treatment. Each CMA_3 group's morphologic characteristics are represented in Table I.

Table I

Sperm characteristics (mean±SD) of 140 patients using four CMA_3 staining groups (95% confidence interval of the mean)

	CMA_3 categories			
	Group A <44%IVF **(n=15)**	**Group B** ≥44%-60%IVF **(n=39)**	**Group C** ≥60%IVF **(n=45)**	**Group D** ≥60%ICSI **(n=41)**
% normal spermatozoa	26.9%±7.5 (22-31%)	13.6%±6.7 (11-15%)	7.7%±7.5 (5-10%)	4.0%±3.1 (3-5%)
Range for CMA_3 staining (%)	39%±3 (38-41%)	52%±4 (50-53%)	70.9%±9 (68-73%)	80.9%±9 (77-83%)
Number of cases <4% (% normal forms)	0	0	16 2.1%±1.2 (1.5-2.7%)	23 2.1±1.1 (1.7-2.7%)
Number of cases ≥4% (% normal forms)	15 26.9%±7.5 (22-31%)	39 13.6%±6.7 (11-15%)	29 12.1%±7.4 (9-15%)	18 8.0±2.1 (7-9%)
Sperm count ($x10^6$/ml)	84.2±31.0 (66-101 $x10^6$/ml)	60.3±29.5 (50-69 $x10^6$/ml)	49.5±33.5 (39-59 $x10^6$/ml)	17.0±20.2 (10-23 $x10^6$/ml)
% Motile cells	65.3%±17.2 (55-73%)	57.9%±12.1 (53-61%)	65.6±10 (61-69%)	37.9±22 (29-46%)

Sperm morphology and in vitro fertilization results versus CMA_3 staining

Evaluation of the 140 semen analyses revealed 39 cases diagnosed as normozoospermic (≥14% normal forms), 61 cases as teratozoospermic (4-13% normal forms) and 39 cases as severe teratozoospermic (<4% normal forms). As

expected, semen parameters were significantly poorer among the ICSI treated patients (Group D), compared to that recorded of the IVF group (Group A, B & C). The percentage morphologically normal spermatozoa and the sperm count ($x10^6$/ml) were significantly different in the 3 IVF and ICSI Groups. Percentage motility in the four groups did not differ significantly (Table I). Although the highest fertilization rate (Table II) was found in Group A (72.2%), it was not significantly higher than the fertilization rate in Group B (64.0%). The fertilization rates reported for Group C (43.7%) and Group D (35.0%) were significantly lower compared to the fertilization rates in Group A (72.2%) and Group B (64.0%, $p<0.0001$).

In the ICSI-and IVF-groups where CMA_3 staining values of >60% were reported, the fertilization rates were respectively 35.0% and 43.7%. This difference was not statistically significant. The cleavage rates as well as embryo quality in the four different groups were similar Likewise, the number of embryos transferred per patient in the four different groups did not differ (Table II).

Table II

Comparison between fertilization rates of 1032 metaphase II oocytes retrieved from 140 patients and chromatin packaging quality as recorded by CMA_3 staining.

	CMA3 GROUPS			
	A: IVF <44%	B IVF ≥44%- 60%	C: IVF ≥60%	D: ICSI≥60%
Number of oocytes	126	268	281	357
Number of oocytes fertilized	91	171	123	124
Fertilization rates	72%a	64%b	43.7%c	35%d
Cleavage	95%	91%	945	86%
Number of embryos transferred	3.4	3.2	2.8	2.6
	Embryo quality grading (%)			
Grade 1	41	39	46	35
Grade II	41	42	39	40
Grade III	12	39	11	14
Grade IV	6	40	4	10

Paired Fisher's exact test:: a vs. b: $p>0.05$; a vs. c: $p<0.05$; a vs. d: $p<0.05$; b vs. c: $p<0.05$; b vs. d: $p<0.05$

However the implantation - and clinical pregnancy rates of the ICSI-and IVF-groups with a CMA_3 percentage of >60% were significantly lower (Table III). No significant difference were observed in the implantation - and clinical pregnancy rates in the IVF -groups with a CMA_3 percentage of <44% and ≥44% to 60% (Table III).

Table III

Comparison between pregnancy outcome and chromatin packaging recorded among 4 groups of patients

GROUP	IMPLANTATION RATE (%)		Clinical Pregnancy per embryo transfer (%)
	Per embryo transfer	Per aspiration	
A IVF CMA_3 <44%	43a	40e	32i
B: IVF 44<CMA_3<60	35b	33f	28j
C: IVF CMA_3 <60	29c	21g	18k
D: ICSI CMA_3>60	20d	17h	15l

Paired Fisher's exact test:: a vs. b: $p>0.05$; a vs. c: $p<0.05$; a vs. d: $p<0.05$; b vs. c: $p<0.05$; b vs. d: $p<0.05$; e vs. f:: $p>0.05$; e vs. g: $p<0.05$; e vs. h: $p<0.05$; f vs. g: $p<0.05$; f vs. h: $p<0.05$; i vs.j: $p>0.05$; i vs k: $p<0.05$; i vs. l: $p<0.05$; j vs k: $p<0.05$; j vs. l: $p<0.05$. :

For IVF patients we used a fertilization rate cut off value of 60% and a CMA_3 staining percentage of 44% and calculated the predictive values for CMA_3 as far as fertilization success is concerned. The positive predictive value was 80%, negative predictive value 49%, sensitivity 22% and specificity of 93%. The low negative predictive value (49%) and sensitivity (22%) implies that CMA_3 staining does not have a high discriminate level in predicting fertilization failure. The positive predictive value of 80% and specificity of 93% indicates the power of CMA_3 staining to predict fertilization success. Possible explanation for the low sensitivity is possibly due to the large population of patients (n=39) that revealed slightly elevated CMA_3 (>44%-60%) values, but succeeded in fertilizing >60% of the oocytes. Furthermore, using a CMA_3 cut off value of 60% instead of the

44.5%, the 2x2 contingency table revealed a sensitivity of 49%, specificity of 91%, PPV of 80% and NPV of 60%.

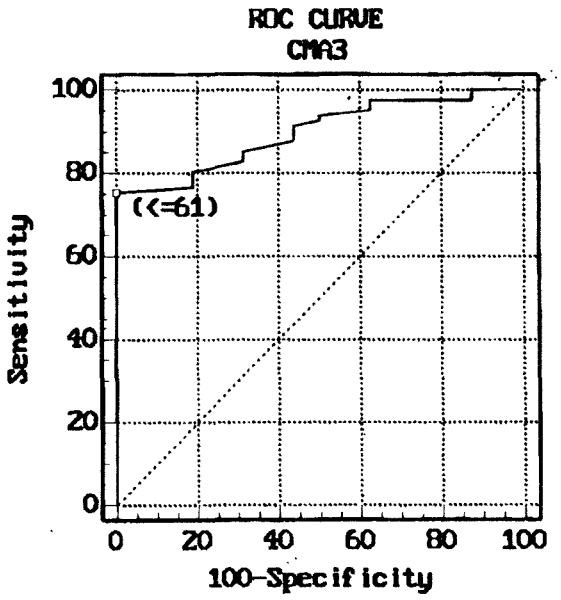
ROC curve analyses

The discriminating power of nuclear maturity, as recorded by CMA_3 staining, to identify abnormal morphology values and fertilization failure in the IVF groups, were calculated with Receiver Operator Characteristics (ROC) analyses.

During ROC analyses we used 4% normal forms as a cut off value to calculate the optimum sensitivity and specificity levels for CMA_3 staining. The areas under the curve were 0.89, sensitivity 75% and specificity 100%. During these calculations, the indicated cut off value for CMA_3 staining, to distinguish between <4% and ≥4% morphology groups, was 60% (Figure 1).

Figure 1

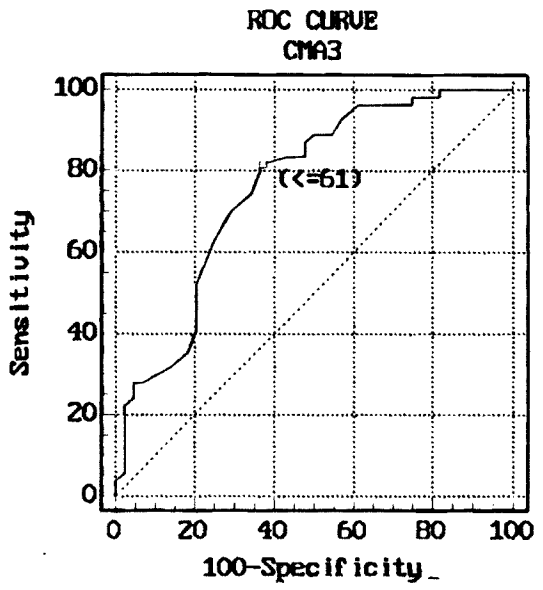
Receiver Operator Characteristics analyses of CMA_3 staining and percentage normal sperm morphology >4% and <4% normal forms. Optimal prediction of correct morphologic classification occurred at CMA_3 values of >61%.



When in vitro fertilisation rates of >60% and <60% were used in the ROC analysis, the optimal CMA_3 value for accurate prediction of fertilization success was again recorded at 60%. The area under the curve was 0.76, sensitivity of 81.5% and specificity of 63.6%. (Figure 2).

Figure 2

Receiver Operator Characteristics analyses of CMA_3 staining and in vitro fertilization rates of >60% and <60%. Optimal prediction of in vitro fertilization occurred at CMA_3 values of >61%.



DISCUSSION

The development of a sequential analytical programme in the diagnostic andrology laboratory, apart from sperm functional tests, should include assays that provides information that will assist clinicians on the therapeutic approach of couples attending an assisted reproductive programme. During a Consensus Workshop in Advanced Andrology (ESHRE Special Interest Group) four categories of tests have been proposed as important sperm functional tests (i) computer-assisted evaluation of sperm motion characteristics (CASA), (ii) inducibility of the acrosome reaction, and bio-assays that sequentially assess

gamete interaction including (iii) sperm-zona pellucida binding tests and (iv) sperm-hamster egg penetration assay (7)). Different laboratories have highlighted the diagnostic power of these tests and the World Health Organization (WHO) has incorporated them under the category of functional tests (8). Therefore, additional techniques underlying fertilization failure or success prior to IVF or ICSI would be an important clinical tool for clinicians. It is vital to understand the choreography of fertilization by ICSI, how it might differ from events that occur during IVF, and how dissimilarities between ICSI and IVF might raise clinical concerns (17).

The ability of sperm to fertilize is not only closely correlated with the its morphology (2), but also to the quality of the chromatin packaging (10, 11). When CMA_3 staining is used as a discriminator of in vitro fertilisation success (>60% oocytes fertilized) a sensitivity of 81.5% and specificity of 63.6% was recorded. The 95% confidence interval did not include 0.5 (0.70 to 0.83), meaning that CMA_3 staining could distinguish between IVF success and failure. An area under the curve of 0.76 means that a randomly selected individual with CMA_3 staining of >60%, will fertilise >50% of metaphase II oocytes in 76% of the cases.

CMA_3 staining seems to be excellent discriminator of sperm morphology, since a sensitivity, that is the probability that the percentage CMA_3 staining will be elevated (>60%) in the presence of poor sperm morphology, was calculated at 75%. The specificity, that is the probability that CMA_3 staining will reflect

decreased staining values (<60%) among the sperm morphology group >4% normal forms, was 100%. Again, the 95% confidence interval did not include 0.5 (0.81 to 0.90), meaning that CMA_3 staining could distinguish between >4% and <4% morphology samples. A total of 39 (28%) in the study was diagnosed as severe teratozoospermic. Group A and B i.e. patients with ≤44% and ≥44%-60% CMA_3 respectively, had no severe teratozoospermic (<4% normal forms) patients. On the other hand groups C and D, i.e. men with >60% CMA_3 staining, contained 16 and 23 men, respectively, with severe teratozoospemic semen. The CMA_3 cut off value be influenced according to the morphological quality of the sperm samples used in the patient populations. Each laboratory should determine its own cut off value for CMA_3 staining and the results should be interpreted with caution since the cut off value of one institution might be totally different from the other. Its is therefore important to stratify the data from a large patient population in order to identify the grey areas of the test.

In addition, the decrease fertility reported here is not only associated with sperm displaying abnormal morphology, but also revealed an increased sensitivity of the DNA to denaturate (18). These observation have been confirmed (19, 9) whereby morphological abnormal sperm showed both a high level of fluorescence to CMA_3 fluorochrome and a high presence of endogenous nicks in the DNA. Even though good sperm morphology data have been correlated with fertility success, a normal shaped sperm head may contain chromosomes with microdeletions, aneuploidy, DNA strand breaks, and abnormal sperm chromatin

structure and yet fertilizes an oocyte. On the other hand since ICSI overrides deficiencies in sperm motility, zona and oolemma binding and leaves the successful completion of fertilisation upon the sperm nucleus, we suggest that chromatin packaging assessments should be included as a complementary assay to the sequential diagnostic approach of the male factor patients (20, 21).

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Clinical importance of zona pellucida induced acrosome reaction (ZIAR): predictive value of the ZIAR test for in vitro fertilization.

Human Reproduction 2000, submitted

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ABSTRACT

The study aimed to establish zona pellucida induced acrosome reaction response (ZIAR) among 35 couples with normal and G-pattern sperm morphology and repeated poor fertilization results during assisted reproduction treatment. ZIAR tests were performed using 0.25 zona pellucida/ml co-incubated with spermatozoa for 60 minutes. Acrosome reactions were measured with FITC-PSA staining, and expressed as the difference between stimulated and unstimulated (spontaneous) spermatozoa populations. Results were compared with in vitro fertilization rates of metaphase II oocytes. Interactive dot diagrams divided the patients into 2 groups i.e. ZIAR<15% and ZIAR>15% with mean fertilization rates of 49% and 79%, respectively. The sensitivity and specificity for ZIAR results versus fertilization were 93% and 100%, respectively. The area under the curve was 99% and the 95% confidence interval did not include 0.5 that implies that the ZIAR test is able to predict fertilization failure among IVF patients. In

conclusion, the ZIAR test has diagnostic potential since it can assist the clinician to identify couples that will benefit from ICSI therapy.

Introduction

The astounding success rates achieved by intracytoplasmic sperm injection (ICSI) (Van Steirteghem et al., 1993), emphasised the need to refine sperm functional evaluation. This is particularly true in cases of profound male factor infertility and contemporary andrology laboratories therefore should be able to select the most appropriate form of treatment for each couple, especially those diagnosed as male factor infertility (Kruger and Coetzee, 1999; Oehninger et al., 1991, Oehninger et al., 1997). Moreover, the past 10 -15 years has brought not only an explosion in the number of laboratory tests for human sperm functions, but also the belief among many clinicians that sperm function testing is now irrelevant due to the advances in IVF technology. Ideally, an accurate and inexpensive test would be used to determine which men require ICSI and which do not. The current success with ICSI has stifled rather that stimulated the search for such a test. Also the history of abuse of sperm functional tests has not helped; far too many have been heralded by their advocates as the best diagnostic approach and clinicians have been too quick to pronounce men fertile or infertile on the basis of a single favourite test.

At a Consensus Workshop in Advanced Andrology (Consensus Workshop 1996) it was suggested that because of their validation and unquestioned clinical value, the homologous sperm-zona pellucida binding tests should be incorporated in the advanced stages of the work-up. Sperm-zona binding

reflects multiple sperm functional events depicting sperm-zona pellucida interaction i.e. completion of capacitation as manifested by the ability to bind to the zona pellucida and to undergo ligand induced acrosome reaction. A diagnostic test underlining the ability of the spermatozoa to undergo the acrosome reaction in response to homologous zona pellucida would be a valuable additional tool in the male fertility workup schedule (Franken et al., 1990, Oehninger et al., 1992, Liu and Baker 1992).

During the consensus workshop it was agreed that better standardisation of the currently used acrosome reaction techniques should be implemented prior to their introduction as a routine clinical tool. The present report is an effort towards developing and standardisation of the acrosomal response as a clinical tool in the assisted reproductive programme. The most widely utilised method is the acrosome reaction (AR) ionophore challenge test (ARIC), during which the acrosome reaction is induced by calcium ionophore and then identified with defined by lectins in combination with indirect immunofluorescence (Consensus Workshop, 1996). The rationale for the development of this test was manifested in the precise timing of acrosomal response. (Cummins et al., 1991; Tesarik, 1989; Tesarik, 1996). Guidelines for the interpretation of the acrosome response during the ARIC test defined AR pre-maturity in cases where >20% of spermatozoa show spontaneous AR after 3-h incubation under capacitating conditions. AR was reported to be normal if >15% difference existed among spontaneous and induced AR; <10%

difference is abnormal and indicates a possible impairment of fertilization (WHO Laboratory Manual, 1999).

The ARIC test as well as the concept of acrosomal inducibility (Henkel et al., 1993, Henkel et al., 1998) was used as a predictor of sperm fertilizing ability as compared with tests that simply measure the frequency of spontaneous AR. The ZP, both intact and solubilized, has been demonstrated to be a powerful and physiological inducer of the acrosome reaction (Cross et al., 1988, Bielfeld et al., 1994, Liu and Baker 1994, Florman et al., 1989, Franken et al., 1996, Franken et al., 1997). During fertilization, acrosome reaction failure can be caused by multiple factors; such as (i) inadequate sperm capacitation; (ii) an inability of the sperm membrane to undergo specific structural-functional changes after binding to the ZP; or (iii) an impaired capacity of the ZP of a specific oocyte to induce the acrosomal cascade.

The study aimed to evaluate and establish among non-male-factor couples with repeated "poor" or no fertilization during in vitro fertilization treatment;

1. Their acrosome responsiveness (AR) to solubilized human zona pellucida, the results will be used to define;

1.1 *insufficient acrosome response* i.e. where the difference between spontaneous and zona induced AR is less that <15%,

1.2 *normal AR response* where the difference between spontaneous AR and induced AR is ≥15%.

Material and Methods

Patients

All patients in the study signed an informed consent form after Institutional Review Board approval was obtained. The inclusive criteria for patients accepted into the study were (1) females diagnosed with tubal factors, (2) having a normal FSH/LH ratio on day-3 of the menstrual cycle (<10U/L), (3) producing three or more pre-ovulatory metaphase II oocytes at retrieval (4) having husbands with normal sperm parameters as well as normal and g-pattern (4--14% normal forms) morphology and (5) a history of repeated poor (<30% metaphase II oocytes fertilized) fertilization or complete fertilization failure during previous cycles. The mean (±SD) age and number of oocytes retrieved per patient was 30±3 years and 8.3±2 oocytes, respectively. A total of 35 from 382 (9%) couples adhered to the above inclusive criteria.

Ovulation-Induction

Ovulation-induction protocols were adapted to individual needs. Thirty-three patients in the study were treated with a "short" protocol, while two who did not respond well on the short protocol, were treated with the "long" protocol. The long protocol started with 0.5mg/day Buserelin (GnRHa, Suprefact, Hoechst, IHD, Johannesburg, Republic of South Africa) or Lucrin (GnRHa, Abbott, IHD, Johannesburg, Republic of South Africa) on day 21 of the menstrual cycle and continued until the day of hCG administration. E2 is checked 14 days after the first GnRHa injection. If E2 levels are below 100pg/ml, the GnRHa dose is reduced thereafter to 0.2mg/day until hCG injection. Patients are given 225IU

(3 ampoules) Gonal-F (recombinant FSH, Serono, Pty (Ltd), Centurion, Republic of South Africa) daily for 6 days. In the short protocol patients received 0.3mg GnRHa and Gonal-F (3 ampoules) at the same time on day 2 of the cycle. These hormones are administered daily until two dominant follicles of 18mm diameter are ready on ultrasound, after which ovulation is triggered with 10 000 IU hCG (Profasi, Serono, Pty (Ltd), Centurion, Republic of South Africa) administration.

Oocyte evaluation

Metaphase I and II oocytes were identified according to their nuclear maturity status. In metaphase II oocytes a first polar body was present while in metaphase I oocytes no polar body was observed. Following oocyte retrieval great care was taken to ensure the evaluation and classification of the oocytes, were meticulously carried out by the embryologist. Under microscopic vision, metaphase II oocytes were characterised by its round, even shape and presence of light colour and homogeneous granularity. It was always associated with an expanded, luteinized cumulus and a "sun-burst" corona radiata. The membranes of the granulosa cells harvested along with the metaphase II oocytes are also luteinized, loosely aggregated and have mature features (Veeck, 1988)

In vitro fertilization

Metaphase II oocytes were cultured as suggested in the IVF Science Product Manual (Granberg, 1999). Shortly, culture dishes (Falcon 1006, Becton Dickinson SA Scientific, Randburg, Republic of South Africa) were prepared

using 24 hours pre-equilibrated culture media (IVF-50; Scandinavian IVF Science products, Gothenburg, Sweden) and Universal IVF medium; Medi-Cult, MØllehaven, Jyllinge) and Sigma mineral oil (Sigma-M-8410, embryo tested, Sigma-Aldrich, Johannesburg, Republic of South Africa). Three-ml IVF-50 medium was incubated in tissue culture grade plastic tubes (Falcon 2058, SA Scientific, Randburg, Republic of South Africa) for the final swim-up. Spermatozoa were washed in ASP-100 (Scandinavian IVF Science products, Gothenburg, Sweden) and layered with 1,5ml IVF-50 medium. Oocyte insemination was performed with standard sperm concentration of 100 000 motile sperm/mL/oocyte in 3 mL IVF-50 culture medium. On day one, oocytes were denuded and evaluated by the embryologist for fertilization status.

Rescue Intra cytoplasmic sperm injection (RICSI)

The oocytes of patients where no fertilization was reported one day after aspiration were subjected to RICSI. Prior to injection the unfertilized oocytes were placed in 10µl HEPES buffered droplets under oil using 1006 Falcon Petri – dishes. RICSI was performed as a therapeutic procedure and before the ZIAR results were available. In short, a small volume (2-3µl) of prepared spermatozoa was deposited in a droplet of Polyvinylpyrrolidone (PVP, Cat No 10890001, Medicult, Harrilabs, Randburg, Republic of South Africa). Narishige Micromanipulators mounted on a Nikon Inverted Microscope were used to perform the sperm injection. The oocyte was stabilised by applying negative suction to a holding pipette (K-HPIP-1035-5, Cook, Queensland, Australia) An individual morphologically normal sperm was immobilised and

sucked into a Cook injection pipette (K-MPIP-1035-5, Cook, Queensland, Australia). The sperm was injected into the ooplasma and the injected oocyte was then incubated according to the IVF protocol.

Preparation of zonae pellucidae

Solubilization of zona pellucida was performed using 20 oocytes at a time depending on the number of ZIAR tests to be performed. This method was developed during previous reports and results revealed it to be optimal in our laboratory, since (i) the small volumes of solubilized zonae can be stored (7-10 days) at 4°C (Franken et al., 1996, Franken et al, 2000), thus avoiding loss of precious zona material and (ii) since the ZIAR tests were performed blindly on the day of the oocyte retrieval, the use of small volumes of zonae was more practical . Unpublished data from our laboratory showed that the acrosome inducing potential of the different zona pellucida batches did not differ between batches. Unfertilized oocytes (no pronuclei or second polar body) donated by diagnosed male factor couples from the in vitro fertilization programme were used to induce the AR in the study. Oocytes with <10 spermatozoa attached to the zona pellucida were rinsed in Dulbecco's Phosphate Buffered Saline (DPBS, D-5773, Sigma Aldrich, Midrand. Republic of South Africa). Twenty oocytes were placed in 0.1ml DPBS in an Eppendorf 0.5ml micro tube (Micro Tubes, Safe-Lock, Eppendorf-Netheler-Hinz-GmbH, Hamburg, Germany) at 4^0C. The oocytes were washed by centrifugation for 10 minutes at 2600rpm. Using a stereoscope, DPBS was then gently aspirated with a glass drawn Pasteur micropipette. DPBS was added and oocytes were

centrifuged again. Twenty microliters Acid Tyrode's (T-1788, Tyrode's Solution Acidic, Embryo tested, Sigma Aldrich, Vorna Valley, Republic of South Africa) solution was gently added to the oocyte pellet after removal of the DPBS. The zonae were subsequently dissolved at room temperature (45-60 min) in Acid Tyrode's by agitating the zonae solution every 5 minutes with a glass drawn Pasteur Pipette. The reaction was neutralised, by adding 20µl 1mM NaOH. Zonae solutions with a concentration of 0.5 zona pellucida per microlitre (0.5ZP/µl) were stored up to two weeks at 4^0C (Franken et al., 2000). Previous reports indicated adequate acrosome reaction stimulation occurred using microvolumes of sperm and solubilized zona pellucida. During these studies serial dilutions of disaggregated human zona pellucida i.e. 2.5, 1.25, 0.6 and 0.3 ZP/µl induced 32%, 26%, 18% and 14% sperm to acrosome react. Spontaneous acrosome reaction values were 10% (Franken et al., 1996, Franken et al 2000).

Preparation of spermatozoa

The semen from 10 fertile sperm donors (14 samples) were used, during a pilot study, as controls to establish the baseline acrosome reaction response among a fertile population, not attending an assisted reproductive programme. Spermatozoa from 35 patients taking part in our IVF programme with normal and G-pattern morphology were used. Liquefied semen was washed in ASP-100 (Scandinavian IVF Science products, Gothenburg, Sweden) for 10 minutes at 1700rpm. ASP-100 medium is a modified human tubal fluid culture medium containing standard concentrations of 25mM sodium bicarbonate

supplemented with 10mg/ml human serum albumin. The sperm pellet was layered with 1.5ml IVF-50 medium (Scandinavian IVF Science Products, Gothenburg, Sweden) and incubated for 30 minutes at 37^0C and 5% CO_2 to achieve a swim-up separation of motile cells. After the incubation period, 1.0ml of the top sperm suspension was aspirated, a 0.2ml portion of highly motile sperm was then removed and placed in a plastic tube (Falcon 2058, Becton Dickinson, SA Scientific, Randburg, Republic of South Africa) for the Zona induced acrosome reaction (ZIAR). This fraction was then washed once at 1700rpm for 10 minutes with DPBS and the pellet was resuspended in 100 µl DPBS, whilst the remaining volume was used for insemination purposes in the IVF laboratory.

Zona Induced Acrosome Reaction Test (ZIAR-test)

Prior to the study technician and sample variation was recorded, by establishing intra- and inter assay/technician coefficient of variations for PSA-FITC staining. Intra-assay and technician variation was determined by evaluating 100 cells on 5 different microscopic fields (total 500 cells) from the same semen specimen by each of three technicians. Inter- assay and technician variation was accomplished by evaluating staining by counting 100 cells on slides prepared from 5 different specimens by the same sperm donor. Coefficient of variations (CV) for both intra-and inter assay and intra-and inter technician values were calculated by dividing the mean with standard deviation X100% for each observation. The inter-and intra assay as well as inter-and intra technician coefficient of variation was <15% and <10% among

the slides and three technicians, respectively. Results were discarded in cases where the CV's for assay and technician exceeded 15% or 10%.

Twenty microliter (20 µl) of the zonae solution and 20 µl prepared sperm ($5x10^6$ sperm/ml) were mixed in a microplate (Greiner, Lab and Scientific Equipment, North Riding, Republic of South Africa). The mixture was aspirated into a Hamilton Pipette Tip (R84254, Hamilton, Separations, Republic of South Africa) using a 1.0ml sterile, non-pyrogenic latex free syringe (Becton Dickinson, SA Scientific, Randburg, Republic of South Africa). The tip containing the sperm-zonae solution was incubated in a Falcon dish (Falcon 3003, Becton Dickinson, SA Scientific, Randburg, Republic of South Africa) for 60 minutes at 37^0C and 5% CO_2. After the incubation period the spermatozoa were expelled onto the glass slide and coded "test" sperm, while "control" sperm were aspirated from the DPBS resuspended sample described above. Pilot studies have indicated NaOH neutralized acid Tyrode's solution had similar effect on the acrosome reaction compared to that recorded with DPBS, and we therefore used DPBS to resuspend the control. Slides were air dried and fixed in 100% ethanol for 24 hours. Thereafter the spermatozoa were stained for 2 hours at room temperature with 30µg/ml PSA (*Pisum sativuma* agglutinin) labelled with fluorescein-isothiocyanate FITC (L-0770, Sigma Aldrich, Vorna Valley, Republic of South Africa). Finally, slides were washed in DPBS and mounted with DPBS. A minimum of 100 spermatozoa were counted under a Nikon fluorescent microscope (Labophot 2, Nikon, IMP, Johannesburg, Republic of South Africa), Filter Ex 465-495 0 with 400X

magnification. The following staining patterns were evaluated as acrosome reacted spermatozoa; (i) patchy staining on acrosomal region, (ii) distinct staining in the equatorial region occurring as a equatorial bar and (iii) and no staining observed over entire sperm surface. Since we used only swim up sperm samples, the motility was in all cases >80%. Acrosome reaction data presents the findings recorded for live sperm. Spermatozoa with patchy FITC-PSA staining were classified as a population of sperm where the acrosome reaction was initiated and all were classified as acrosome reacted. The acrosome reaction results were evaluated on the day of oocyte aspiration, in order to ensure the AR results to be recorded blindly by the technicians. The zona induced acrosome reaction was calculated as the difference between zona induced AR minus the spontaneous (unstimulated) AR results (A minus B, Table I).

Statistical Analyses

The diagnostic accuracy of the zona induced acrosome reaction result's was illustrated with the interactive dot diagram, while the positive and negative predictive values were recorded with the Receiver Operating Characteristics (ROC curve) (Altman and Bland, 1994, Schoonjans et al., 1995).

Results

The acrosome responsiveness (mean±SD) obtained from fertile sperm donors are depicted in Table I.

Table I

Acrosome reaction and semen analyses of 10 fertile (14 samples) patient population (mean ± SD, range)

Stimulated AR (Test) (0.25ZP/ul)	Unstimulated AR (Control)	% ZIAR	Sperm parameters		
AR (%) **A**	AR (%) **B**	**A - B**	Sperm conc. (10^6cells/ml)	Motility (%)	Morphology (% normal)
36.6±3.2 (29-41)	14.8±2.7 (11-20)	21.3±2.6 (18-25)	58.8±12 (45-85)	60.0±4 (50-70)	17.7±3 (14-25)

The zona induced acrosome reaction as well as the IVF results of the 35 couples are depicted in Table II. The mean sperm concentration, percent normal cells and percent motile cells were 60.2±4, 12.7±0.6 and 58.8±2, respectively.

Table II

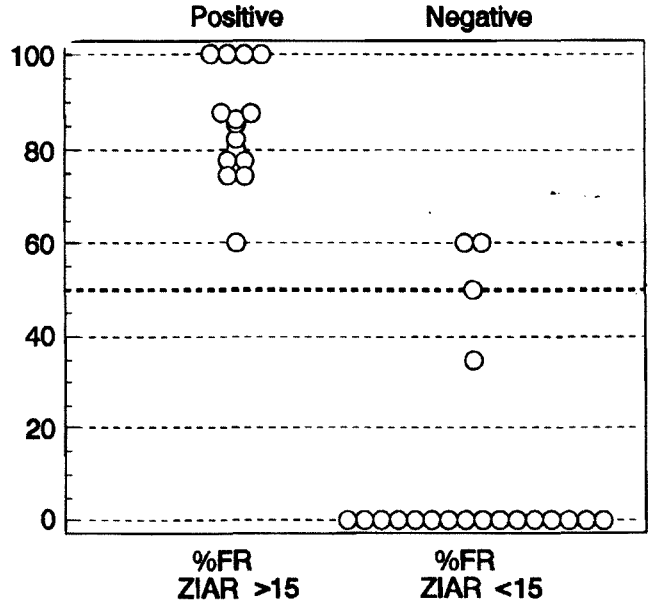
Acrosome reaction and in vitro fertilization results of 35 couples attending assisted reproductive programme (mean±SD).

Stimulated AR (Test) (0.25ZP/ul)	Unstimulated AR (Control)	ZIAR (%)	In vitro fertilization			
AR (%) **A**	AR (%) **B**	**A - B**	# oocytes retrieved	# fertilized	# divide	% fertilized
27.0±2 (7-49)	15.4±1 (5-46)	12.8±2 (1-27)	8.5 ±5.4 (2-22)	3.3±0.6 (0-10)	3.0±0.5 (0-10)	42.4±7 (0-100)

The correlation coefficient between the ZIAR and fertilization rates was r=0.95 (p=0.0001). The interactive dot diagram (Figure 1) illustrates the diagnostic power of ZIAR.

Figure 1

The interactive dot diagram of ZIAR results and in vitro fertilization rates reported for metaphase II oocytes



This value was chosen at 15% based on the following reasons (i) shifting the cut off points for zona induced AR (from 7-19%) during the ROC analyses the 15% level had optimum sensitivity and specificity namely, 60% and 68%, respectively for morphology and 100% and 90%, respectively for fertilization rates (ii) furthermore, the mean ZIAR value was recorded at 12.8±2%, and using 1XSD above the mean i.e. 14.8% which was rounded to 15% and (iii) there was a clear cut in the data at 15%,

The in vitro fertilization rates were calculated as the total fertilization rate (total number of pre-ovulatory oocytes fertilized/total number of pre-ovulatory

oocytes inseminated) at 88.0 ± 19% (mean ± SD). The minimum total fertilization rate (mean minus 2XSD) that can be considered normal in our IVF program is therefore 50%. Total fertilization values below 50% are considered to be abnormal. From the ROC analyses, the optimum sensitivity and specificity was calculated at 50% for the best separation between the two groups ZIAR>15% and ZIAR <15%.

A cut off value for the fertilization rates of 50% was chosen for the best separation between the two groups ZIAR>15% and ZIAR <15%. The corresponding sensitivity and specificity was 93% and 100%, respectively. Using the interactive dot diagram as guide we then divided the couples according to the ZIAR test outcome. The mean (±SD) sperm parameters, acrosome response of the ZIAR groups >15% and <15% are depicted in Table III. The mean (SD) sperm concentration, percent normal cells and percent motile cells were $55.8x10^6$ cells/ml, 12.9±4% and 54.5±13%, respectively for the group with <15% ZIAR values. For the ZIAR group >15% the sperm concentration, percent normal cells and percent motile sperm were 66.4X106 cells/ml, 12.6±3 and 64.7±11, respectively.

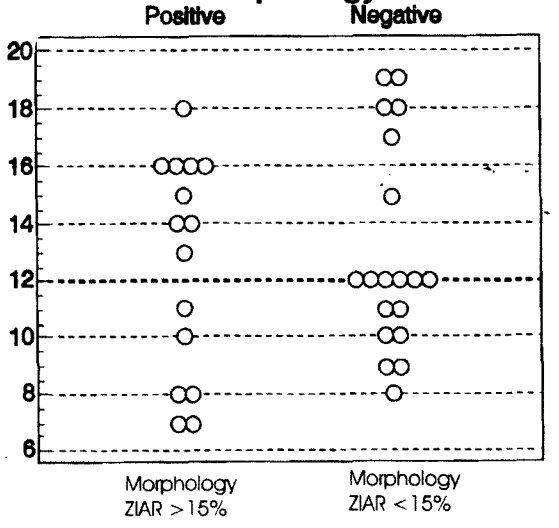
Table III
Acrosome response and IVF outcome of couples divided according to the ZIAR test results (mean±SD, range)

	Stimulated AR (Test) (0.25ZP/ul)	Unstimulated AR (Control)	ZIAR (%)	In vitro fertilization results of metaphase II oocytes			
ZIAR Group	AR (%) **A**	AR (%) **B**	**A - B**	# oocytes	# fertilized	# divide	% fertilized
<15% n=15	21.6±11a (8-35)	17.9±13c (6-40)	3.7±3e (0-8)	8.9±6 (2-22)	0.8±2g (0-2)	0.7±1l (0-2)	49.2±41k (0-100)
>15% n=20	34.4±5b (31-49)	12.2±5d (8-42)	22.2±3f (16-27)	8.2±4 (4-18)	6.7±3h (0-10)	6.3±2j (0-10)	79.6±16l (0-100

Unpaired t-test: a vs. b: p=<0.001; c vs. d: p>0.05;
e vs. f: p=<0.001; g vs. h: p=<0.001; i vs. j: p=<0.001; k vs. l: p=<0.001

Due to the design of the study no correlation could be established between normal morphology and fertilization rates. The interactive dot diagram (Figure 2) illustrates a sensitivity of 60% and specificity of 68%.

Figure 2
The interactive dot diagram of ZIAR results and spermatozoa morphology.



These results were expected since normal morphology was an inclusive criterion for the study.

Rescued intracytoplasmic sperm injection (RICSI)

The oocytes (n=146) of 16 couples were used during a RICSI procedure after fertilization failure was reported in the IVF laboratory. The results of the ZIAR test, as well as the fertilization data during IVF and RICSI are presented in Table IV. The mean (±SD) sperm concentration, percent normal morphology and percent motility were 54.8±17x10^6 cells/ml, 12.6±0.2 and 53.5±1, respectively.

Table IV

The ZIAR and in vitro fertilization for Metaphase II oocytes and RICSI results of 16 couples with fertilization failure

Stimulated AR (Test) (0.25ZP/ul)	**Unstimulated** AR (Control)	**% ZIAR**	**# oocytes**	**RICSI**		
AR (%) **A**	AR (%) **B**	**A – B**		# fertilized	% fertilized	# divided
20.6±12	17.3±13	3.3±2	9.0±6.8	3.5±0.8	39.8±28	3.3±3

The fertilization rate achieved during RICSI was 39.8±28%. Retrospectively, the ZIAR test results i.e. 3.3±2%, were indicative of fertilization failure among these couples. During the following cycle, two couples from this group were prospectively referred to the ICSI programme account of their ZIAR data as well as the fertilization history. The oocytes retrieved were randomly divided to undergo either IVF or ICSI treatment. During this cycle, the retrieved oocytes were randomly allocated to undergo either IVF or ICSI treatment. A total of 21 and 12 oocytes were retrieved from these patients, of which 11 and 6 respectively, were allocated to the IVF programme. The rest of the oocytes,

namely 10 and 6, respectively received ICSI treatment. Complete fertilization failure was again reported for both patients during IVF, while 7/10 (70%) and 4/6 (66%) fertilization rates were reported during the ICSI treatment.

DISCUSSION

Substantial information concerning the mammalian sperm acrosome reaction (AR) and its relevance during fertilization processes has accumulated during the past few years (Brucker and Lipford 1995, Bielfeld et al., 1994, Liu and Baker 1994, Franken et al., 1996, Franken et al., 1997, Liu and Baker 1996a). The data includes information on the mechanism of the AR, the role in the fertilization process, the characterization of AR abnormalities compromising fertility, and methods whereby these abnormalities can be diagnosed and treated (Liu and Baker 1996a, Liu and Baker 1996b). Recent studies have evaluated the nature of the receptors involved in the response and the way AR-inducing signals are transduced from the receptors to the membrane fusion effectors responsible for the ensuing exocytotic reaction (Luconi et al., 1998). Progesterone and zona pellucida glycoprotein (ZP3) have been identified as natural oocyte-associated AR-inducing ligands, and their sequential action has been shown to underlie the physiological AR (Melendrez *et al.* 1994; Roldan *et al.*, 1994, Franken et al., 1997).

The ARIC test identifies two types of AR pathology, namely, 'AR insufficiency' (Tesarik and Mendoza, 1993) and 'AR prematurity' (Tesarik and Mendoza 1995). AR insufficiency describes cases in which the difference in frequency

of AR between ionophore-treated and untreated aliquots of a capacitated sperm population is <15%, while AR prematurity define cases in which the frequency of spontaneous AR is >20% (Tesarik and Mendoza, 1995). Both of the mentioned pathologies can be occur in the same patient. AR insufficiency as revealed by the ARIC test, can only reflect downstream anomalies of the calcium influx in the signal transduction cascade responsible for AR induction (Tesarik 1996).

During the present study we defined normal AR in cases where the zona mediated induction (ZIAR) was significantly correlated with in vitro fertilization and ZIAR outcome was >15%. Insufficient AR was identified in cases where ZIAR was <15% and typically associated with fertilization failure. The percentage of sperm among the group of men showing an insufficient AR also had an elevated spontaneous AR of 17.9%. These findings i.e. patients with normal conventional semen analyses, failure to fertilization with standard IVF and low ZIAR test results may have a similar condition described by Liu and Baker (1994). Here they reported on 10 couples with longstanding infertility presenting reduced frequency of the acrosome reaction of spermatozoa bound to the zona pellucida. They concluded in that study the possibility of the existence of a zona bound sperm population with a disordered acrosome reaction thus causing impaired fertility (Liu and Baker 1994). In close agreement with our findings, Liu and Baker (1997) concluded that patients with disordered ZIAR can be treated by ICSI. Patients with a long history of idiopathic infertility or complete fertilization failure should be tested for this

condition using sperm-zona pellucida interaction tests. Reduced ZIAR patients should be directed to ICSI rather that standard IVF.

Tesarik and Mendosa (1995) described a "premature" AR which might be an indication of dysfunctional capacitation processes leading to a "premature" AR manifesting in an increase number of spontaneous acrosome reacted cells. We were unable to identify a 'premature AR" group in our study since the ZIAR<15% group did not statistically differ between stimulated and spontaneous AR's i.e. 21.6±11% and 17.9±13% ($p>0.05$, unpaired t Test). There was also no difference between normal controls (14.8±2.7, Table I) and those patients with low ZIAR (17.9±13, Table III).

Important to note that the in the ZIAR >15% group, the percentage normal sperm was 12.9±4% (95% confidence interval, 11.2 to 14.6%). The reported fertilization rates among men with 5-14% normal spermatozoa and in those cases where no corrective measurements were taken (increase insemination concentration) are 64%. (Kruger et al., 1986). The ZIAR test therefore, identified a sperm population that has normal sperm parameters, with dysfunctional acrosome responsiveness to solubilized human zonae pellucidae. ZIAR test results can be used clinical to distinguish between couples that will benefit from IVF treatment, but more importantly seems to be an indicator for ICSI therapy. The sixteen couples with complete fertilization failure during IVF of 146 metaphase II oocytes and who were treated with a RICSI procedure (Table IV), had a decreased ZIAR of 3.3±2% (mean±SD). ZIAR results in these cases could possibly be seen as a powerful indicator of

fertilization failure. On account of the ZIAR results, the metaphase II oocytes of two couples from RICSI group, who did not achieve success during the first treatment period, were randomly divided and used for IVF or ICSI treatment during the following cycle. Both couples failed fertilization during IVF, where a 70% and 66%, fertilization rate respectively for patient 1 and 2 was recorded during ICSI.

The fundamental utility of the ZIAR test stems from its being a functional bioassay of sperm performance in relation to the human zona pellucida. Together with tight binding to the zona pellucida, the ZIAR potential of a sperm population can be seen as a requisite for in vitro fertilization. The ZIAR results provide useful discrimination between men capable of achieving fertilization in vitro versus those who are unlikely to be successful. The results can become a valuable tool in the diagnostic scheme of the consulting clinician, since the outcome of the test underline the most effective clinical option, namely IVF or ICSI.

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The clinical importance of a micro-assay for the evaluation of sperm acrosome reaction using homologous zona pellucida.

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ABSTRACT

The study aimed to develop an acrosome reaction (AR) assay using micro-volumes of solubilized human zonae pellucidae among 35 couples. Sperm morphology values were g-pattern (5-`14% normal forms) and normal patterns (>14% normal forms). All couples had repeated poor or failed in vitro fertilization rates form the assisted reproductive programme. A zona induced acrosome reaction (ZIAR) test was performed using homologous 0.25 zona pellucida/ml incubated with sperm to mediate AR. Acrosome reactions were measured with FITC-PSA staining, and expressed as the difference between zona induced and spontaneous AR spermatozoa. Results indicated that microvolumes of solubilized human zona pellucida could successfully be used to determine acrosome reaction status of spermatozoa. The results were compared with in vitro fertilization rates of metaphase II oocytes and expressed with Receiver Operating characteristics (ROC). ROC analyses divided the patients into 2 groups i.e. ZIAR<15% and ZIAR>15%. The

correlation coefficient between ZIAR and in vitro fertilization was r= 0.94 ($p<0.0001$). The sensitivity and specificity for ZIAR results versus fertilization were 93% and 100%, respectively. The present results can be used as an clinical indictor for fertilization failure, thus assisting clinicians to refine the therapeutic approach of infertile couples prior to the onset of the treatment.

Ejaculated spermatozoa are unable to fertilize the oocyte unless they undergo an activation process, called capacitation, and a morphological change, called the acrosome reaction. Capacitation occurs during the transport of the spermatozoa through the female genital tract and involves a biochemical alteration in the anterior sperm membranes. Once these membrane changes have occurred, the spermatozoa can initiate the acrosome reaction when it approaches the zona pellucida (Fraser, 1995, Austin, 1951, Austin, 1952). When cells are fully capacitated, they can express hyperactivated motility which provides a positive forward thrust to aid in the penetration of the oocyte (Yanagimachi, 1994).

During sperm penetration through the follicle cell layers (immediately before and after binding to the zona pellucida), stimulation of plasma membrane receptors by one or more oocyte components (e.g. zona pellucida protein ZP_3) takes place. (Fraser, 1995). Receptor stimulation activates signal transduction pathways, ultimately resulting in the activation of cAMP-, cGMP- and phospholipid dependent protein kinase i.e. protein kinase A, G and C (Zaneveld et al., 1991)

The past 10 -15 years has brought not only an explosion in the number of laboratory tests for human sperm functions, but also a concern among many clinicians that sperm function testing is now irrelevant due to the advances in vitro fertilization (IVF) technology. Ideally, an accurate and inexpensive test would be used to determine which men require intracytoplasmic sperm injection (ICSI) and which do not. The success of ICSI has stifled rather than inspired the research on improving the diagnostics tools available in the andrology laboratory (Van Steirteghem, 1993).

Furthermore, their advocates have maltreated sperm functional tests, since far too many tests have been heralded as the best diagnostic approach and clinicians have been too quick to pronounce men fertile or infertile on the basis of a single favourite test. For example, although sperm morphology has been employed with astounding success as a sensitive indicator of male factor infertility; sperm morphology is not the answer to all the problems encountered in the andrology laboratory. This is especially true in cases where both gametes seem to be normal as far as its microscopic appearance is concerned (Kruger at al., 1986, Veeck 1988). In some of these cases, fertilization failure is repeatedly reported in the IVF laboratory. Important questions often asked generally address the concern whether gamete morphology necessarily measures all sperm functions.

The World Health Organisation has incorporated some of the functional assays under the category of functional tests (WHO, 1999). At a recent Consensus Workshop in Advanced Andrology it was agreed that better standardisation of the currently used acrosome reaction techniques should be implemented prior to their introduction as a routine clinical tool (Consensus workshop, 1996).

In accordance with the recommendations made by the Consensus Workshop on the clinical importance of the acrosome reaction, we developed a micro-assay where the physiological induction of the acrosome reaction in human sperm was validated (Franken et al., 2000). The methodology of the assay facilitated the use of minimal volumes of solubilised, homologous zona pellucida. This newly devised micro-technique is easy and rapid to perform, is repeatable and facilitates the use of minimal volumes of solubilized human ZP (even a single ZP) for assessment of the inducibility of the acrosome reaction of a homologous sperm population. The present study aimed to evaluate the ability of the micro-assay for the determination of the human zona induced acrosome reaction (ZIAR), to predict fertilization failure.

Material and Methods

Preparation of solubilized zonae pellucidae

Unfertilized oocytes (no pronuclei or second polar body) donated by diagnosed male factor couples from the in vitro fertilization programme, were used to induce the AR in the study. Oocytes with <10 spermatozoa attached to

the zona pellucida were rinsed in Dulbecco's Phospate Buffered Saline (DPBS, D-5773, Sigma Aldrich, Midrand. Republic of South Africa). Twenty oocytes were placed in 0.1ml DPBS in an Eppendorf 0.5ml micro tube (Micro Tubes, Safe-Lock, Eppendorf-Netheler-Hinz-GmbH, Hamburg, Germany) at 4^0C. The oocytes were washed by centrifugation for 10 minutes at 2600rpm. Using a Nikon stereoscope (Nikon, SMZ-10, IMP, Boksburg, Republic of South Africa) DPBS was then gently aspirated with a glass drawn Pasteur micropipette. Acid Tyrode's (T-1788, Tyrode's Solution Acidic, Embryo tested, Sigma Aldrich, Vorna Valley, Republic of South Africa) was added and oocytes were centrifuged again prior to the addition of 20µl Acid Tyrode's. The zonae were dissolved at room temperature (45-60 min) in Acid Tyrode's by agitating the zonae solution every 5 minutes with a glass drawn Pasteur Pipette. The reaction was neutralised, by adding 20µl 1mM NaOH to the solution. Zonae solutions with a concentration of 0.5 zona pellucida per microlitre (0.5ZP/µl) were stored up to fourteen days at 4^0C.

Micro -assay for Zona Induced Acrosome Reaction Test (ZIAR-test)

Twenty microliters (20 µl) of the zonae solution and 20 µl prepared sperm ($3x10^6$ cells/mL) were mixed in a micro-titerplate (Greiner, Lab and Scientific Equipment, Republic of South Africa). The mixture was aspirated into a Hamilton Pipet Tip (R84254, Hamilton, Separations, Republic of South Africa) using a 1.0ml sterile, non-pyrogenic latex free syringe (Becton Dickinson, SA Scientific, Randburg, Republic of South Africa). The tip containing the sperm-

zona solution was placed horizontally in a humidified, pre-warmed Falcon dish (Falcon 3003, Becton Dickinson, SA Scientific, Randburg, Republic of South Africa) for 60 minutes at 37^0C and 5% CO_2. After the incubation period spermatozoa were expelled onto the glass slide and coded "test" sperm, while "control" sperm were aspirated from the DPBS resuspended sample described above. Slides were air dried and fixed in 100% ethanol for 24 hours. Thereafter the spermatozoa were stained for 2 hours at room temperature with 30µg/ml PSA (*Pisum sativum* agglutinin) labelled with fluorescein-isothiocyanate FITC (L-0770, Sigma Aldrich, Vorna Valley, Republic of South Africa). Finally, slides were washed in DPBS and mounted with DPBS. In all cases 100 spermatozoa were counted under a Nikon fluorescent microscope (Labophot 2, Nikon, IMP, Johannesburg, Republic of South Africa), Filter Ex 465-495 0 with 400X magnification. The following staining patterns were evaluated as acrosome reacted spermatozoa; (i) patchy staining on acrosomal region, (ii) distinct staining in the equatorial region occurring as a equatorial bar and (iii) and no staining observed over entire sperm surface. Non-reacted spermatozoa showed a bright yellow stained acrosomal cap. Sperm motility values were assessed directly following incubation and prior to the acrosome preparation to ascertain the percentage live/dead spermatozoa in the samples. Motility recordings were done by assessing the percentage motile sperm under phase contrast microscopy (Nikon-TMS, IMP, Boksburg, Republic of South Africa). Since all sperm samples were retrieved after wash and swim-up separation, motility values were in all cases >80%.

Preparation of spermatozoa

To establish AR values of a fertile population, semen from 10 fertile sperm donors (14 samples) were used as controls for acrosome reaction studies during a pilot study. Sperm samples were collected from 35 patients attending our IVF-programme. A 20-minute liquefaction period was allowed prior to the washing period. Semen samples were washed twice in ASP-100 (Scandinavian IVF Science products, Gothenburg, Sweden) for 10 minutes at 1700rpm. The sperm pellet was layered with 1.5ml IVF-50 medium (Scandinavian IVF Science products, Gothenburg, Sweden) and incubated for 30 minutes at 37^0C and 5% CO_2 to achieve a swim-up separation of motile cells. After the incubation period, the top 1.0ml sperm suspension was aspirated and the sperm concentration was adjusted to $1.5x10^6$ cells/mL. A portion (200µl) of the swim up volume containing highly motile sperm was placed in a Falcon 2058 tube (Becton Dickinson, SA Scientific, Randburg, Republic of South Africa) to be used during the zona induced acrosome reaction test (ZIAR). This fraction was then washed at 1700rpm for 10 minutes in DPBS after which the pellet was resuspended in 100 µl DPBS (sperm concentration $3x10^6$/mL). Twenty microliters of this suspension was used in the ZIAR test. The remaining volume from the swim up sample was used for insemination purposes in the IVF laboratory.

Ovulation-Induction

Ovulation-induction protocols were adapted to individual needs. Buserelin (Suprefact, Hoechst, IHD, Johannesburg, Republic of South Africa), Lucrin (GnRHa, Abbott, IHD, Johannesburg, Republic of South Africa), and Gonal-F (recombinant FSH, Serono, Pty (Ltd), Johannesburg, Republic of South Africa) were used in the so-called "long" protocol. The "long" protocol treatment was induced in the mid-luteal or late luteal phase whilst the "short" protocol administration of GnRHa begins in the early follicular phase. Both treatments continued until the day of HCG (Profasi, Serono, Pty (Ltd), Johannesburg, Republic of South Africa) administration. FSH (Metrodin or Gonal-F, Serono, Pty (Ltd), Johannesburg, Republic of South Africa) treatment was started after desensitization was achieved. The dose of FSH was individualised. When the largest follicle reached a diameter of 18mm and the Estradiol serum levels indicated a satisfactory follicular response, 10 000 IU HCG was administered.

Oocyte evaluation

Following oocyte retrieval, the embryologist meticulously carried out the classification and evaluation of the oocytes. Under the microscope, oocytes were characterised by its round, even shape, opaque colour and homogeneous granularity. It was always associated with an expanded luteinized cumulus and a "sun-burst" corona radiata. The membranes of the granulosa cells harvested along with the metaphase II oocytes are also luteinized, loosely aggregated and have mature features Metaphase I and II

oocytes were also identified according to their nuclear maturity status. In metaphase II oocytes a first polar body was present while in metaphase I oocytes no polar body was observed (Veeck, 1988).

In vitro fertilization

Metaphase II oocytes were cultured as suggested in the IVF Science literature (24). Shortly, culture dishes (Falcon 1006, Becton Dickinson GmbH, Heidelberg) were prepared using 24 hours pre-equilibrated culture media (IVF-50; Scandinavian IVF Science products, Gothenburg, Sweden) and Sigma mineral oil (Sigma-M-8410, embryo tested, Sigma-Aldrich, Johannesburg, Republic of South Africa). Three-ml IVF-50 medium was incubated in Falcon 2058 tubes (Becton Dickinson GmbH, Heidelberg) for the final swim-up. Spermatozoa were washed in ASP-100 (Scandinavian IVF Science products, Gothenburg, Sweden) and layered with 1,5ml IVF-50. Insemination and oocyte culturing took place in IVF-50. On day one oocytes were denuded and fertilization assessed.

Statistical Analyses

The diagnostic accuracy of the zona induced acrosome reaction and positive and negative predictive values were recorded with the Receiver Operating Characteristics (ROC curve) (Altman and Bland, 1994). The relationship between ZIAR data and IVF was recorded as correlation coefficient.

Results

The sperm parameters and acrosome responsiveness (mean±SD) obtained from fertile sperm donors were: sperm concentration; 58.8±12x10^6 cells/ml, motility; 60.0±4(% live) and morphology 17.7±3 (% normal, strict criteria). The mean percentage AR sperm recorded during ZIAR was 21.3±2.6%.

The ZIAR was calculated as the difference between zona induced AR (stimulated) minus the spontaneous (unstimulated) AR results (A minus B, Table 1).

Table1

Acrosome reaction and in vitro fertilization results of 35 couples attending assisted reproductive programme (mean±SD).

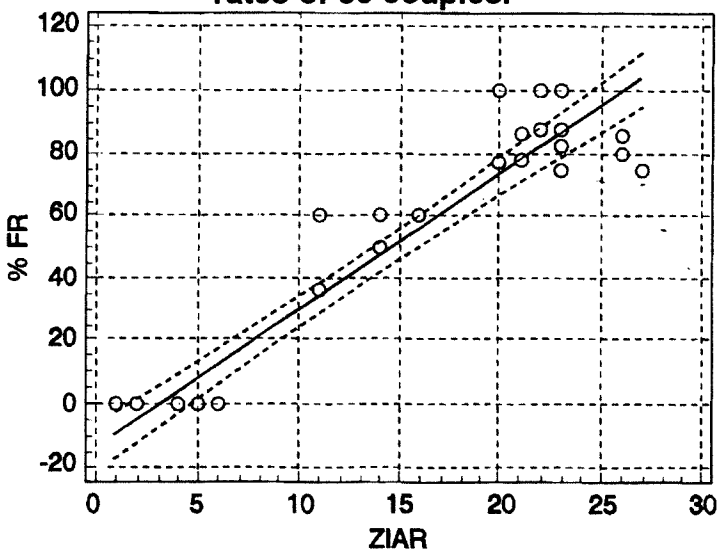
Stimulated AR (Test) (0.25ZP/ul)	Unstimulated AR (Control)	ZIAR (%)	In vitro fertilization			
AR (%) **A**	AR (%) **B**	**A – B**	# oocytes retrieved	# fertilized	# divide	% fertilized
27.0±2	15.4±1	12.8±2	8.5 ±5.4	3.3±0.6	3.0±0.5	42.4±7

Similar to the guidelines set for the ARIC test, the data were allocated to one of the following AR categories; *insufficient acrosome response* i.e. where the difference between spontaneous and zona induced AR is less that <15%, *normal AR response* where the difference between spontaneous AR and induced AR is ≥15%.

The semen parameters, zona induced acrosome reaction as well as the IVF results of the 35 couples are depicted in Table 1.

The correlation coefficient between the ZIAR and fertilization rates was r=0.95 (Figure 1, p=0.0001). The receiver operating characteristics (ROC) representing the correlation between ZIAR, in vitro fertilization rates and sperm morphology are depicted in Figure 1. In order to evaluate the clinical importance, a ZIAR cut off value of 15% was chosen i.e. a difference between zona induced AR minus spontaneous AR of 15%. This value was arbitrary chosen at 15% since there was a clear cut in the data at 15%, furthermore the mean AR value was recorded at 12.8%, which we rounded of to 15%. A cut off value for the fertilization rates of 50% was chosen for the best separation between the two groups ZIAR>15% and ZIAR <15%. The corresponding sensitivity and specificity was 93% and 100%, respectively.

Figure 1
Correlation between zona induced acrosome reaction and fertilization rates of 35 couples.



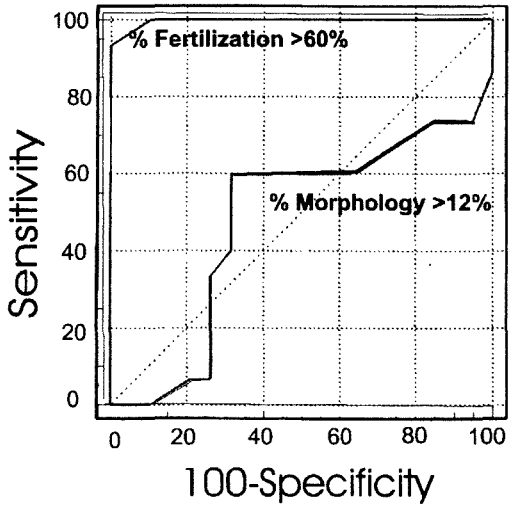
Due to the experimental design of the study no correlation could be established between normal morphology and fertilization rates. This was expected since normal morphology was an inclusive criterion for the study.

ROC curve analyses

The discriminating power of the ZIAR to identify fertilization failure among the 35 couples, were calculated with Receiver Operating Characteristics curve (ROC) analyses. The power of the ZIAR (>15% and <15% acrosome reacted) of a given sperm sample to predict the fertilization is represented in Figure 2.

Figure 2

Receiver Operator Characteristics (ROC) of the zona induced acrosome reaction data, fertilization rates and percentage normal cells of 35 couples from an IVF programme



The area under the ROC curve, for the ZIAR data of 99% illustrates the predictive power of the test. An area under the curve of 0.99 implies that a

randomly selected individual with ZIAR result of <15%, will have fertilization failure in 99% of cases. The confidence interval (0.89 to 1.00) does not include 0.5, implying that ZIAR results have the ability to distinguish between fertilization rates of >50% and <50%.

DISCUSSION

The AR is a receptor-mediated cellular response and therefore many recent studies have investigated the nature of the receptors involved in the response and the way AR-inducing signals are transduced from the receptors to the membrane fusion effectors responsible for the ensuing exocytotic reaction. *Progesterone* and a highly conserved *zona pellucida glycoprotein* termed ZP_3 have been identified as natural oocyte-associated AR-inducing ligands, and their sequential action has been shown to support the occurrence of the physiological AR (Melendrez *et a*l. 1994; Roldan *et al.*, 1994).

There are also apparent divergences between the two pathways because the one used by the zona pellucida ligand involves a pertussis toxin-sensitive G protein (Franken et al., 1993), whereas that used by progesterone does not (Tesarik et al., 1993). Progesterone reacts with a multi-receptor system on the sperm surface and this system co-operates with that used by ZP_3 to control the physiological AR.

At the present time, there seems to be general agreement that more clinical information can be gained by the analysis of a stimulant-induced acrosomal exocytosis compared to the assessment of the spontaneous frequency of acrosome reactions. The most widely utilised method is the acrosome reaction ionophore challenge test (ARIC test) where the acrosome reaction is identified with defined lectins in combination with indirect immunofluorescence (Cummins et al., 1991). The AR frequency in capacitated sperm populations is simply correlated to the sperm fertilizing potential and it is now clear that the AR must be precisely timed to ensure fertilization (Tesarik, 1989). A premature AR leads to the loss of zona pellucida recognition sites from the sperm surface and thus compromises sperm-zona pellucida binding (Liu and Baker, 1994, Franken et al., 1996).

With the use of the ARIC test two types of AR pathology have been defined: 'AR insufficiency' (Tesarik and Mendoza, 1993) and 'AR prematurity' (Tesarik and Mendoza 1995). AR insufficiency describes cases in which the difference in frequency of AR between ionophore-treated and untreated aliquots of a capacitated sperm population is <15%, while AR prematurity is used for case in which the frequency of spontaneous AR is >20% (Tesarik and Mendoza, 1995); both pathologies can be occur in the same patient.

The zona pellucida (ZP), both intact and solubilized, has been demonstrated to be a powerful and physiological inducer of the acrosome reaction (Cross et

al., 1988, Bielfeld et al., 1994, Liu and Baker 1994, Franken et al., 1996, Franken et al., 1997). However, in diagnostic laboratory practice, the physiological acrosome inducer i.e. zona pellucida is not readily used. This is true, since good correlation has been found between the ARIC test and fertilization rates, but mainly because human zonae are unavailable in adequate quantities for routine use. While several groups are working on the production of recombinant human ZP3 (rhuZP3), it is not yet available in a biologically active form and in significant amounts. The opinion is however, that rhuZP3 will be the ultimate agonist or trigger substance for human sperm AR, and will form the basis of the "perfect acrosome reaction test" (Tesarik 1996).

The newly described assay is simple, can be performed quickly, and the results are reliable and repeatable. Therefore, because of the small volumes employed, it is an ideal technique for testing native and recombinant ZP (highly precious or scant material). The need for a micro-volume assay to assess acrosome reaction has been identified previously (Morales and Cross, 1989). Micro-volumes are necessary due to the fact that diagnostic andrology laboratories often lack sufficient biological material (i.e., human ZP) to perform a defined and specific test such as the examination of the physiological acrosome reaction. This is true for the natural ZP protein(s), but will also be relevant when recombinant human ZP proteins are to be tested for corroboration of their biological activity (Brewis et al., 1996, Chapman and

Barratt, 1996). The results of the present study indicated the use of a single ZP to be adequate for mediating the acrosome reaction of a sperm population in suspension.

The routine introduction of a simple and reliable assay for the evaluation of the physiologically-induced acrosome reaction as a component of the previously proposed sequential diagnostic work up programme (Oehninger et al., 1991, Oehninger et al., 1997), will assist in the identification of specific sperm defects and may allow the development of more directed therapies. Andrology testing remains, in our opinion as well as those of others (Mortimer 1994), an ever-growing component in the work up of the infertile couple.

From the results depicted in the ROC curve and in close agreement with the guidelines set for the ARIC test, a "normal" ZIAR result was calculated at ≥15%, while insufficient AR was accordingly defined as those sperm samples where the ZIAR was <15%. The ZIAR test has the ability to identify a sperm population with normal sperm parameters (motility, morphology and concentration), with impaired acrosome responsiveness to solubilized human zonae pellucidae. The results can be used in a clinical setting to distinguish between couples that will benefit from IVF treatment, but moreover seems to be an indicator for ICSI therapy. The fundamental utility of the ZIAR test stems from its being a functional bioassay of sperm performance in relation to the human zona pellucida. The ZIAR results served as a clinical indictor and

discriminate successfully between men capable of achieving fertilization in vitro versus those who are unlikely to be successful.

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Defective sperm decondensation: a cause for fertilization failure.

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Summary

The study aimed to evaluate the role of chromatin packaging (CMA_3 staining) and sperm morphology during sperm-zona binding, sperm decondensation and the presence of polar bodies among oocytes that failed in vitro fertilization (IVF). The percentage CMA_3 staining categorized the data in three groups, <44%, n=10; ≥44-59%, n=10; and ≥60%, n=29, Morphology groups were ≤4%, (n=11); >4-14% (n=19); and >14% (n=19). One hundred and seventy out of a total of 225 oocytes that failed IVF were evaluated for sperm-zona binding, ooplasma penetration and sperm decondensation. Odds ratio analyses indicated that being in the CMA_3 staining group >60%, increases the risk of decondensation failure 15.6 fold relative to CMA_3 staining <44%. For morphology the risk of fertilization failure decreases 2.17 fold in the morphology group with normal cells >4-14%, while it increases 2.45 fold for morphology-group with normal cells ≤4%. Using CMA_3 fluorescence to discriminate, 51% of the oocytes in the group with elevated CMA_3 fluorescence, had no sperm in the ooplasma compared to 32% and 16% penetration failure in the CMA_3 staining groups ≥44-59% and <44%,

respectively. Sperm chromatin packaging quality and sperm morphology assessments are useful clinical indicators of human fertilization failure. The use of immunofluorescence techniques could be used in order to provide a clear diagnosis of failed fertilization.

Introduction

Assessment of spermatozoa of patients undergoing in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), often reveal distinct differences in the mean percentage of spermatozoa presenting normal morphology, Chromomycin A_3 (CMA_3) fluorescence (an indicator of the packaging quality of sperm chromatin) and endogenous nicks (an indicator of DNA damage in the sperm nucleus). This implies that men with normal sperm morphology would present a CMA_3 fluorescence of <44% (Esterhuizen *et al.,* 2000, Franken *et al.,* 1999) and endogenous nicks in <10% of their spermatozoa (Sakkas *et al.,* 1998). An association between abnormal chromatin packaging and the presence of DNA strand breaks has been shown to exist and it has been postulated that these anomalies may arise due to faults in the mechanisms that package and protect the sperm chromatin during spermatogenesis (Manicardi *et al.,* 1995, Sailer *et al.,* 1995, Nikolettos *et al.,* 1999).

Despite the continuous improvement of in vitro fertilization technologies, fertilization failure is a recurrent phenomenon, explained mainly in terms of chromosome alterations. Several studies focussing on oocytes analyzed by cytogenetic techniques showed different types of fertilization failure after

conventional in vitro fertilization (Plachot and Grozet, 1992). Although explanations in terms of chromosomal alterations are well documented the participation of sperm chromatin packaging, sperm decondensation, resumption of meiosis, oocyte activation and pronuclei migration has been less well studied. Different aetiologies for fertilization failure in humans have been described by as well as the absence of oocyte activation described among injected oocytes during ICSI treatment (Asch *et al.,* (1995). Further, the spermatozoon may remain poorly accessible to oocyte factors required for chromatin decondensation and formation of the female pronucleus (Tesarik and Kopecny, 1989a, Tesarik and Kopecny 1989b).

Immunofluorescence technology has made it possible to analyze stages at which human fertilization fails and the intracellular cell communication with the oocyte after penetration can be traced. The role of the spermatozoon in this process can be studied with precision as well as the visualization of the oocyte after sperm penetration has occurred. (Hewitson *et al.,* 1999, Schatten *et al.,* 1989, Simerly *et al.,* 1998). Although, couples presenting with fertilization failure during IVF can be treated with ICSI, the diagnosis of the causative factors is important. There are many reports on the relationships between sperm factors and fertilization rates in vitro, but it remains difficult to determine the frequency of various factors contributing to failure of fertilization (Liu and Baker, 2000).

The present study aimed to record the role of chromatin packaging, sperm morphology, sperm-zona binding, sperm head decondensation, presence of

polar bodies among oocytes that failed fertilization during in vitro fertilization treatment.

Material & Methods

Three hundred and ninety seven (397) oocytes were obtained from 49 patients treated for infertility in an *in vitro* fertilization programme. Forty-nine couples selected for the study all a history of repeated poor (<30% metaphase II oocytes fertilized) fertilization or complete fertilization failure during two previous cycles. One hundred and seventy two (172 i.e. 43%) oocytes were reported fertilized while 225 (57%) failed fertilization during the cycle of investigations. From the 225 oocytes that failed to fertilize 170 were stained with (2'-[Ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole) Trihydrocloride (Hoechst 33342 Sigma, Cat B-2261, Sigma Chemicals, St Louis, MO USA). Fifty-five oocytes could not be used for Hoechst 33342 staining due to degeneration during in vitro culture. The Hoechst staining data of the oocytes were compared with CMA_3 staining percentage normal cells in the semen. All semen evaluations reported here were recorded on the sample processed for IVF.

Patients

All patients in the study signed an informed consent form after Institutional Review Board approval was obtained. The inclusive criteria for patients accepted into the study were (1) females diagnosed with tubal factors, (2) having a normal FSH/LH ratio on day-3 of the menstrual cycle (<10U/L), (3) producing three or more pre-ovulatory metaphase II oocytes at retrieval (4) having husbands with normal sperm parameters as well as normal and g-pattern (4–14% normal forms) morphology and (5) a history of repeated poor

(<30% metaphase II oocytes fertilized) fertilization or complete fertilization failure during previous cycles. The mean (±SD) age of the females was 30±3 years.

Ovulation-Induction

The ovulation induction protocol was individualized according to indications and the infertility history of the patient. Ovarian stimulation protocol included the use of a GnRHa (gonadotrophin-releasing hormone agonist), Buserelin (Suprefact, Hoechst, IHD, Johannesburg SA), Lucrin (Abbott, IHD, Johannesburg, SA) or and the subsequent addition of hMG or FSH (Metrodin, Serono, Pty (Ltd), Johannesburg, SA), Gonal-F (Serono, Pty (Ltd), Johannesburg, SA) treatment after desensitization was achieved. Exogenous gonadotropins were administered daily until the lead follicles averaged between 18mm and 20mm in diameter, as measured by ultrasound and the estradiol serum levels indicated a satisfactory follicular response. 10 000IU Human chorionic gonadotropins hCG (Profasi, Serono, Pty (Ltd), Johannesburg, SA) administration was administered 36 – 37 hours prior transvaginal oocyte recovery.

Oocyte evaluation

Metaphase I and II oocytes were identified according to their nuclear maturity status. In metaphase II oocytes a first polar body was present while in metaphase I oocytes no polar body was observed. Following oocytes retrieval great care was taken to ensure the evaluation and classification of the oocytes, which were meticulously carried out by the embryologist. Under microscopic vision, metaphase II oocytes were characterised by its round, even shape and presence of light colour and homogeneous granularity. It was

always associated with an expanded cumulus and a "sun-burst" corona radiata. The membranes of the granulosa cells harvested along with the metaphase II oocytes are also luteinized, loosely aggregated and have mature features (Veeck, 1988).

Semen preparation

Spermatozoa were washed in ASP-100 (Scandinavian IVF Science products, Gothenburg, Sweden) and layered with 1,5ml G1.1 culture medium (Scandinavian IVF Science products, Gothenburg, Sweden). A total of 49 semen samples were obtained from the husbands of couples attending our fertility program. After complete liquefaction at room temperature, a basic semen analysis was performed according to the WHO guidelines (WHO, 1999). Semen volume, concentration spermatozoa, motility and forward progression were recorded. Two slides were prepared from each semen sample namely, a slide to record normal spermatozoa (Kruger *et al.,* 1986) and a second slide was used to determine chromatin packaging quality (Bianchi *et al.,* 1996, Sakkas *et al.,* 1996)

The quality chromatin packaging was determined using Chromomycin A_3 staining (CMA_3, Sigma Chemicals, St Louis, MO USA Cat 2659). Smears were prepared (Bianchi *et al.,* 1996, Sakkas *et al.,* 1996), air-dried and fixed in methanol/acetic acid 3:1. After fixation in methanol/acetic acid (3:1) at room temperature for 20 minutes, the slides were once again air-dried. These slides were then stained with 60-100ul CMA_3 in a dark chamber for 20 minutes. Slides were washed in McIlvaine's buffer (Geigy Scientific Tables 1984) and mounted using Dabco (Aldrich Chemicals Co, Milwaukee, USA Cat No.

29,073-4). Spermatozoa were evaluated under a Nikon fluorescent microscope (CFWN10X IMP. Johannesburg, South Africa; Filter Fx 465-495).

In vitro fertilization

Gametes and embryos were cultured in Falcon 1006 culture dishes (Falcon 1006, Becton Dickinson GmbH, Heidelberg) in pre-equilibrated G1.1 culture media (Scandinavian IVF Science products, Gothenburg, Sweden). Culturing procedures took place under embryo tested Sigma mineral oil (Sigma-M-8410, Sigma-Aldrich, Johannesburg, SA). For insemination and oocyte culturing purposes G1.1 was used. After 16 – 18 hours the oocytes were inspected for signs of fertilization. Fertilized oocytes were transferred to 50ul droplets G1.1 - (Scandinavian IVF Science products, Gothenburg, Sweden). The following day the embryos were transferred to dishes, containing fresh 50ul droplets of G1.1. Oocyte insemination was performed with standard sperm concentration of 100 000 motile sperm/mL/oocyte in 3 mL IVF-50 culture medium. Culturing of day 3 embryos (8-10 cells), morulae and blastocyst transfers took place in G2.2 - (Scandinavian IVF Science products, Gothenburg, Sweden).

Preparation of oocytes for fluorescence microscope

The oocytes were examined for signs of fertilization (2 pronuclei and 2 polar bodies) after 18, 48, and 72 hours post insemination. A total of 170 out of 225 oocytes that were evaluated as unfertilized were included in the study. These oocytes were washed three times in 50ul Dulbecco's PBS (DPBS) (Sigma D5773), under embryo tested mineral oil (Sigma M-8410). Staining took place in 20ug/ml droplets Hoechst 33342 (Sigma, B-2261) at room temperature for

60 minutes. Oocytes were rinsed in three changes of DPBS and mounted on a pre-cleaned glass slide. The oocytes were gently compressed under 22X22mm cover slip, supported on the corners by petroleum jelly pillars. Slides were stored at 4°C in a humidified container in the dark and scored in a blinded fashion without prior knowledge fertilization rates reported for the cycle. Stained oocytes were examined using a Nikon Labophot Fluorescent Microscope with a 100W arc bulb. Hoechst 33342 staining was observed through a triple band filter (FITC, Rhodamine and DAPI). The DNA and H33342 complex was excited with UV-light.

Observations were reported for spermatozoa and oocytes using the following six parameters;

percentage normal spermatozoa, chromatin packaging quality, sperm tightly bound to the zona pellucida, sperm penetrating the ooplasma, sperm heads showing signs of decondenstion, decondensed sperm heads, oocytes with 1 polar body oocytes with 2 polar bodies.

Statistical Methods

Logistic regression was employed to model decondensation in terms of CMA_3 and morphology, where decondensation was binary (success or failure). CMA_3 was categorised into 3 groups (<44%, 44-59%, ≥60%) and morphology was also categorised into 3 groups (≤4%, 4-14%, >14%). The reference categories were success for decondensation, <44% for CMA_3-Group C and >14% for morphology-Group C. Positive and negative predictive values were calculated with chi-square test. Group comparisons for the different

parameters were performed with Fishers' exact t-test, chi -square test and Welch T-test where applicable.

RESULTS

The data were analyzed according (i) to the percentage normal sperm as evaluated by strict criteria as well as (ii) chromatin packaging quality as recorded by CMA_3 staining. Semen samples classified the different sperm morphology values reported into ≤4% normal forms, >4%-14% normal forms and >14% normal forms (Kruger *et al.,* 1986). In vitro fertilization results i.e. number of oocytes used, number of oocyte fertilized and cleavage information were stratified according to the above mentioned morphology classification. (Table 1).

Table 1

Fertilization and cleavage results of 397 oocytes divided according to the percentage normal morphology

	Total	**Morphology ≤4% (n=11 couples)**		**Morphology >4%-14% (n=19 couples)**		**Morphology >14% (n=19 couples)**	
		n	Mean ±SD	n	Mean ±SD	n	Mean ±SD
Total number of oocytes inseminated	397	96	8.7±3	139	7.3±3	162	8.5±3
Number of oocytes fertilized	172/397 43%	18/96 (19%)a	1.6±1	58/139 (42%)b	3.0±2	96/162 (59%)c	5.1±2
Number of oocytes cleaved	150/172 87%	16/18 (89%)	1.5±1	52/58 (90%)	2.6±2	82/96 (85%)	4.3±2

Fishers' exact t-test: a vs.b: p=<0.001; b vs.c: p=<0.001; a vs c: p=<0.001

Table 2 represents the results obtained from CMA_3 staining, sperm-zona pellucida binding, ooplasma penetration, sperm head decondensation, and presence of 1 or 2 polar bodies.

Table 2

Sperm characteristics according to sperm morphology groups observed in 225 oocytes that failed in vitro fertilization.

	Morphology ≤4% (n=11 couples)	**Morphology >4%-14% (n=19 couples)**	**Morphology >14% (n=19 couples)**
	Mean±SD	Mean±SD	Mean±SD
Total number oocytes inseminated	96	139	162
Total number of oocytes fertilized	18/96 (19%)	58/139 (42%)	96/162 (59%)
Total number of oocytes with failed fertilization	78/96 (81%)	81/139 (58%)	66/162 (41%)
Total number of oocytes stained with Hoechst 33342	52/96 (54%)	65/139 (47%)	53 (33%)
Mean number of oocytes tested	4.7±2	3.4±2	2.8±1
% normal cells	1.7±0.8a	8.3±3b	23.4±7c
%CMA3	84.5±6e	67.7±14f	48.8±11g
Number of zona bound sperm	13.8±9h	14.4±12i	21.1±6j
Number of ooplasma penetrated	16 (30%)k	46 (72%)l	36 (65%)m
Number of sperm decondensed	9 (56%)n	29 (63%)o	26 (72%)p
Number of oocytes, 1st polar body	46 (87%)	58 (91%)	51 (93%)
Number of oocytes, 2nd polar body	4 (8%)q	19 (30%)r	18 (33%)s

Fishers' exact test: a vs.b p=<0.001, a vs. c: p=<0.0001, b vs. c: p=<0.001
e vs. f p=<0.001, e vs g; p=<0.001, f vs. g: p=<0.001
h vs. j p=<0.001, i vs j; p=<0.001
k vs. l p=<0.001, k vs m; p=<0.001
n vs. p p=<0.001, n vs o; p=<0.001
q vs. r p=<0.002, q vs s; p=<0.002

The criteria for CMA_3 classification were based on the results obtained during a previous study, where 44.5%±13 was reported to be a cut off value for CMA_3 staining among fertile and sub-fertile men (Esterhuizen *et al.,* 2000). This value was similar to that reported by others (Bianchi *et al.,* 1996, Sakkas *et al.,* 1996). In order to analyse the data of the present study; we calculated a second cut off value estimated at 60.0% recorded as 1XSD above 44.5%. Accordingly the data were stratified using 3 basic cut off values for CMA_3 staining, namely <44%, >44 - 59% and ≥60%. In vitro fertilization results such as number of oocytes used, number of oocyte fertilized and cleavage information were analysed according to CMA_3 staining values of 44.5% and 60% as cut off values (Table 3).

Table 3

Fertilization and cleavage results of 397 oocytes divided according to chromatin packaging quality (CMA3).

	Total	**CMA3 ≥60% (n=29 couples)**		**≤44% CMA3 ≥60% (n=10 couples)**		**CMA3<44% (n=10 couples)**	
		n	Mean ±SD	n	Mean ±SD	n	Mean ±SD
Number of oocytes tested	397	235	8.1±3	77	8.3±3	85	8.5±2
Number of oocytes fertilized	172/397 43%	68/235 (29%)a	2.34±2	51/77 (66%)b	5.1±3	53/85 (62%)c	5.3±2
Number of oocytes cleaved	150/172 87%	59/68 (87%)	2.0±2	47/51 (92%)	4.7±2	44/53 (83%)	4.4±2
Number of oocytes with failed fertilization	225/397 57%	167/235 (71%)		26/77 (34%)		32/85 (38%)	

Fishers' exact t-test: a vs.b: p=<0.001; a vs.c: p=<0.001.

Using chromatin packaging as discriminator, the data for rest of the measured observations (i.e. sperm-zona pellucida binding, ooplasma penetration, sperm

head decondensation, and presence of 1 or 2 polar bodies) was again divided into 3 groups namely, CMA_3-Group A (n=29, CMA_3 staining >60%), CMA_3-Group B (n=10, CMA3 staining ≥44-59%) and CMA_3-Group C (n=10, CMA3 staining <44%) (Table 4).

Table 4

Sperm characteristics according to chromatin packaging quality observed in 225 oocytes that failed in vitro fertilization.

	CMA3 ≥60% (n=29 couples)	**≤44% CMA3 ≥59% (n=10 couples)**	**CMA3<44% (n=10 couples)**
	Mean±SD	Mean±SD	Mean±SD
Number of oocyte fertilized	68/172 (29%)	51/172 (66%)	53/172 (62%)
Mean number of oocytes tested	4.2±2	2.5±2	2.5±1
% CMA3	77.5±8%a	49.0±3%b	40.5±4%c
% Normal cells	7.1±6%d	17.3±9%e	24.1±7%f
Number of zona bound sperm	12.3±11g	15.2±11h	35.6±9i
Number of ooplasma penetrated	60 (49%)k	17 (68%)l	21 (84%)m
Number of sperm decondensed	33 (55%)n	15 (76%)o	18 (86%)p
Number of oocytes, 1st polar body	108 (89%)	22 (88%)	25 (100%)
Number of oocytes, 2nd polar body	22 (18%)q	9 (36%)r	10 (40%)s

Fisher's exact t-test: a vs. b p=<0.025, a vs c; p=<0.021
a vs. b p=<0.001, a vs c; p=<0.001, b vs. c p=<0.001
Welch t-test: g vs. j p=<0.001, h vs j; p=<0.001
Fisher's exact t-test k vs. l p=<0.001, k vs m; p=<0.001
n vs. p p=<0.025 n vs o; p=<0.021
q vs. r p=<0.019, q vs s; p=<0.019

The adjusted odds ratios are given in Table 5. Note that the 95% confidence interval's for some of the odds ratios are wide and can be attributed to the variation in the relatively small sample dice to limited availability of suitable subjects. Being in CMA_3-group A (CMA_3 staining >60%), increases the risk of

decondensation failure 15.6 fold relative to CMA_3-group A (CMA<44%), while for CMA_3-group B (CMA_3 staining ≥44-59%) this increase is 11.56 fold. For morphology the risk decreases 2.17 (= 1/0.46) fold for morphology-group B (normal cells >4-14%), while it increases 2.45 fold for morphology-group A (normal cells ≤4%).

Table 5

Odds ratios of risk factors resulting in a lack of decondensation

RISK FACTOR	ODDS RATIO	95% CONFIDENCE INTERVAL
CMA_3-Group A (Morphology <4%)	15.6	(1.19 ; 204.48)
CMA_3-Group B (Morphology ≥4-14%)	11.56	(0.98 ; 136.99)
Morphology- Group A CMA_3>60%)	2.45	(0.26 ; 22.97)
Morphology- Group B (CMA_3 ≥44-59%)	0.46	(0.08 ; 2.49)

Discussion

In order to explore the cellular and molecular aspects of fertilization failure, we studied 172 non - fertilized human oocytes obtained from 49 couples attending the IVF programme. To ascertain whether a relationship existed between sperm chromatin packaging quality, sperm morphology and the ability of spermatozoa to fertilize after IVF, we divided patients according to, the percentage normal spermatozoa as well as the percent CMA_3 fluorescence.

Absence of sperm decondensation and polar body formation was reported the major reason for in vitro fertilization failure after IVF. When failed fertilized oocytes were examined after IVF, using Hoechst 33342 stain, the chromatin

status of the penetrated sperm can be scored as either (i) condensed or (ii) decondensing or decondensed in the ooplasma of metaphase II (one polar body) or telophase (two polar bodies) oocytes or (iii) both paternal and maternal chromatin formed polar pronuclei.

On the other hand, using CMA_3 fluorescence as a discriminator between fertilization success and failure, 51% of oocytes in the group with elevated CMA_3 fluorescence (>60% staining), had no sperm in the ooplasma compared to 32% and 16% penetration failure in the groups with CMA_3 staining ≥44-59% and <44%, respectively. In addition, 45% of the oocytes in the ≥60% CMA_3 group did not show any signs of decondensation, while only 24% and 14% of the spermatozoa in the CMA_3 groups ≥44-59% and <44%, respectively were still in a condensed form present in the ooplasma. These results agree with previously described studies reporting on the lack of oocyte activation to be the most frequent reason observed for fertilization failure among intracytoplasmic sperm injection patients (Lopes *et al.,* 1998). The inability of sperm to decondensate can possibly be explained by the reports of Lopes et al. (1998), who described a significantly higher rate of DNA fragmentation in sperm that failed to decondense compared to decondensed sperm, suggesting a correlation between the rate of DNA fragmentation and loss of oscillin activity (Dozortsev *et al.,* 1995).

When normal sperm morphology was employed as a indicator of fertilization failure, 70% of the oocytes from the severe teratozoospermic group (≤4% normal forms) had no spermatozoa in the ooplasma, while 28% of the

teratozoospermic (>4-14% normal forms) and 35% of the normal group (>14% normal forms) had no sperm. With spermatozoa having morphological impairment but of sufficient quality that they can sometimes penetrate the zona and achieve fertilization, a problem of delayed fertilization and poor embryonic development is evident (Ron-El *et al.,* 1991). The strict criteria for evaluation of human sperm morphology plays an important role in predicting IVF results, and concentrations of progressively motile sperm can be an optional method (Enginsu *et al.,* 1992).

Oehninger *et al.* (1998) investigated the role of the spermatozoon on implantation and pregnancy outcome in IVF/ICSI, in a retrospective, controlled analysis and revealed a trend towards a poorer implantation and pregnancy outcome with IVF, but not within ICSI treatments, among male factor patients. They speculated that poorer results observed among male factor patients undergoing IVF could be associated with the release of toxic factors such as reactive oxygen species (ROS), by abnormal sperm often caused by the high insemination concentration. Toxic factors such as ROS can effect the sperm axoneme as a result of ATP depletion (De Lamirande and Gagnon, 1992) inhibit mitochondrial functions, and synthesis of DNA, RNA and proteins (Comporti, 1989). From previous studies and the present results it seems logic to conclude that once fertilization is achieved, teratozoospermia does not impair embryo quality or viability. Chromatin decondensation disturbance and centrosome anomalies were found to be the major cause for fertilization failure after ICSI (Nikolettos *et al.,* 1999).

Sperm chromatin packaging quality and sperm morphology assessments are useful clinical indicators of human fertilization failure. We therefore suggest that the use of immunofluorescence should be used in order to provide a clear diagnosis of failed fertilization. This will then allow us to understand the aetiology of each particular case in order to take appropriate action in future cycles.

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CHAPTER 4

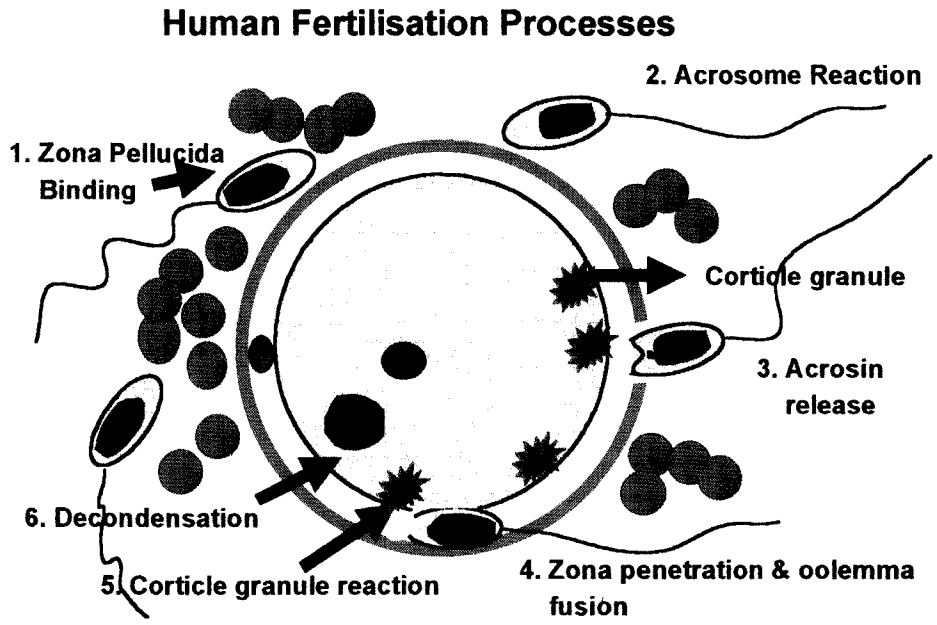
DISCUSSION

Evaluation of sperm has experienced fundamental changes since the advent of assisted reproductive technologies; we understand now, for instance, that sperm function and performance can be improved without major changes in the old semen classification parameters. Although the components of the basic semen evaluation give an approximate idea of the functional competence of the male gamete, a better prediction can be gained from validated sperm functional assays. Still, much work needs to be done to identify and standardize those sperm functions that are more indicative of this competence. For example, the sperm-zona pellucida binding assays have been validated in their ability to predict the results of fertilization in vitro through statistical comparisons using a meta-analytical approach (Oehninger et al., 2000). These tests can immediately be applied to the clinical management of infertile patients within the assisted reproduction setting.

Furthermore, the establishment of predictive tests of sperm function has become a global issue of importance. Since Oehninger et al ,(1995) proposed the instalment of a sequential diagnostic approach as an essential component of the clinical management of the male factor patient, many tests has been heralded by their advocates as crucial for assisted reproductive programmes (Kruger et al.,

1986, Tesarik 1989, Franken et al., 1993, Liu and Baker 2000, Sakkas et al., 1995, Evenson et al., 1999).

Figure 1



During a Consensus Workshop, the participants proposed that the laboratory evaluation of sperm quality/quantity should be approached using a sequential, multistep diagnostic analysis (Figure 1) (Oehninger *et al.*, 1991). It was proposed that this scheme should comprise an initial stage that consists of the basic semen analysis, a second stage involving assessment of some functional and biochemical/enzymatic properties of spermatozoa, and a third stage that includes evaluation of those sperm functions directly related to fertilization, i.e. sperm zona pellucida binding and penetration, sperm-oocyte penetration, nuclear decondensation and assessment of chromatin normality (Oehninger *et al.*, 1991

Oehninger, 1995). The validity of the sequential diagnostic approach is partly highlighted by the present data. The results underlined the value of a multistep diagnostic scheme since we could report on the clinical important role of chromatin packaging quality and zona pellucida mediation of the acrosome reaction on fertilization rates.

Anomalies in chromatin packaging

In both the present studies on chromatin packaging quality (see chapter 3.1 and 3.4) lower fertilization rates were associated with sperm displaying abnormal morphology. These observations have been confirmed by others (Bianchi et al., 1993, Bianchi et al., 1996, Balhorn et al., 1988) whereby morphologically abnormal human spermatozoa show a high level of fluoroscence to CMA_3 fluorochrome and a high presence of endogenous nicks in their DNA. Consistent with these findings is a reduced ability to achieve fertilization in patients with a high percentage of sperm displaying abnormal head morphology, even when micromanipulation techniques are used to assist fertilization. Furthermore, patients that contain a high percentage of sperm with DNA nicks are more likely to show failure in initiating decondensation when their sperm are injected into the cytoplasma of the oocyte using ICSI.

This brings to light the question of what consequences anomalies in sperm chromatin packaging or damaged DNA have during fertilization? In procedure such as ICSI, membrane interactions between the gametes are effectively

transcended, hence the onus for the completion of successful decondensation of the sperm fails largely on a relationship between sperm chromatin organization and the ooplasma. Analyses of the odds ratio in chapter 3.5 indicated that being in the CMA_3 group that showed elevated staining percentages the risk of decondensation failure increased 15.6 fold relative to normal CMA_3 staining i.e. <44%. For morphology the risk of fertilization failure decreases 2.17 fold in the morphology group with normal cells >4-14%, while it increases 2.45 fold for morphology-group with normal cells ≤4%. In this instance it seems as if sperm decondensation impairment was a more sensitive indicator of fertilization failure compared to poor morphology.

Functional integrity of the zona induced acrosome reaction (ZIAR-test)

Up till now the diagnostic laboratory practice was unable to use a physiological inducer of the AR, because human zonae are unavailable in adequate quantities for routine use. While several research groups are working on the production of recombinant human ZP3 (rhuZP3), it is not yet reliably available in a biologically active form in significant amounts. The general opinion, however, is that rhuZP3 will be the ultimate agonist or trigger sbstance for the human sperm AR, and will almost certainly form the basis of the 'perfect acrosome reaction test' of the future (Tesarik 1996).

Here for the first time a selected group of patients that was diagnosed with acrosome pathology on account of the acrosome response to homologous zona

pellucida. Similar to the ionophore challenge (ARIC) test (Cummins *et al.*, 1991), we were able to describe with the zona induced acrosome reaction test (ZIAR test) not only " AR insufficiency", but also "AR prematurity". AR insufficiency identified cases in which the difference in frequency of AR between zona-treated and untreated aliquots of a capacitated sperm population is <15%, while AR prematurity is used for case in which the frequency of spontaneous AR is >15%, both pathologies can be occur in the same patient.

AR insufficiency as revealed by the ZIAR test, can only reflect anomalies situated downstream of the calcium influx in the signal transduction cascade responsible for AR induction. As previously described these events are G-protein regulated (Franken et al., 1996). No abnormality would be diagnosed if the cascade were disturbed selectively at the receptor or post-receptor levels upstream of the calcium influx. To detect such anomalies, the use of methods evaluating the presence or activity of sperm surface receptors, and eventually other signaling molecules, would be required.

Both AR insufficiency and AR prematurity can be treated by intracytoplasmic sperm injection (ICSI) because no relationship between AR function and ICSI results is apparent. The power of the data obtained during the diagnostic cycle (see chapter 3.5) of the two couples are encouraging, since we were able to successfully use the acrosome reaction as a diagnostic tool in the assisted reproductive programme. The addition of ZIAR-test to the sequential analytic schedule would add valuable clinical information as far as the diagnostic

approach is concerned. The power of the test lies in its ability to successfully predict fertilization failure in cases where no male factor was suspected. The fundamental utility of the ZIAR-test stems from its being a functional bioassay of sperm performance in relation to solubilized human zona pellucida.

Our clinical data to date are gratifying, albeit limited to two couples. Thus far, we have found the ZIAR-test to be accurate in predicting fertilization failure. In time we will know whether the ZIAR-test aids in the prediction of pregnancy rates and outcome. Obviously, a much larger and statistically valid data set must be collected prospectively before we can expound on the utilities of the ZIAR-test for predicting the fertilizing potential of a given man.

The present results indicates the importance of a sequential analytical approach in the assisted reproduction arena since the identification of specific sperm defects will allow the development of directed therapies. Andrology testing remains, in our opinion an ever-growing component in the work-up of the infertile couple. Nevertheless, it is imperative that we continue our efforts toward the identification of the specific sperm defects involved as well as their origin (genetic, developmental, environmental or other).

The ICSI setting provides a new unique arena to evaluate sperm dysfunctions at a cellular and molecular level. Consequently, areas that still demand immediate attention are (a) chromosomal and genetic testing (FISH is already being used in order to assess structural and numerical sperm chromosomal deficiencies and

PCR has been introduced for the diagnosis of microdeletions of the Y chromosome); (b) identification of sperm receptors that react with natural ligands (i.e zona pellucida and oolemma) and the signalling cascades that lead to the acrosome reaction and hyperactivated motility, (c) examination of sperm factor(s) involved in egg activation and molecules that mediate cross-talk between egg and sperm after penetration leading to early embryo development, and (d) identification of the regulatory mechanisms and disturbances of spermatogenesis, spermiation and epididymal function (Oehninger et al .,1997).

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Recommendations

The present results indicate the role and importance of additional sperm functional assays during the evaluation of the male partner of couples consulting for infertility. Chromatin packaging assessment was illustrated to be a valuable addition to the sequential diagnostic programme in the assisted reproductive arena, since it correlated significant and positively not only with the percentage normal spermatozoa in the ejaculate, but also with the fertilization rates recorded during in vitro fertilization treatment.

It is recommended that measurement of sperm chromatin packaging quality should be used as a diagnostic test among those couples that are in the assisted reproductive programme, scheduled to undergo either IVF or ICSI treatment. This sperm assessment should be regarded as an additional parameter providing information on fertilization events that occurs post zona binding. It's importance is highlighted by the superior sensitivity, compared to sperm morphology, to indicate decondensation failure.

The data also underline the clinical value of the physiological acrosome reaction. We prospectively examined the acrosome reaction mediated by a single zona pellucida of 10 normal men as well as 35 patients with normal semen analysis and normal or g-pattern morphology. The study introduced the clinical application of the micro-assay acrosome assay thereby underlining important aspects such as the use of small volumes of solubilized zona pellucida to assess the sperm's ability to acrosome react. Technically this feature implies the use of a zona pellucida obtained from a single oocyte. It is recommended that the ZIAR test should be used as a diagnostic test among those couples in an assisted reproductive programme presenting unexpected low fertilization results repeatedly.